Prevalence Phenotypic Characterization of *Listeria Monocytogenes* Isolated from Diseased Sheep

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Abstract

Listeriosis is the third most common food-borne illness and is considered one of the most dangerous bacterial zoonotic infections globally. This research aimed to isolate and characterize *Listeria monocytogenes* (*L. monocytogenes*) from diseased sheep in Egypt. A total of 240 samples collected aseptically from sheep were subjected to isolation and biochemical characterization of *L. monocytogenes*. *L. monocytogenes* are Gram-positive rods that are both aerobic and facultatively anaerobic. Thirty-one listeria isolates (12.9%) were recovered from diseased sheep. All tested isolates were positive for catalase, Voges-Proskauer, carbohydrate fermentation, esculin, gelatin hydrolysis, and methyl red tests. Meanwhile, they were negative for oxidase, indole, urease, and nitrate reduction tests and did not utilize citrate in the citrate utilization test. Listeria isolates produced yellow butt/yellow slant on TSI agar medium with no H_2S or gas production. Therefore, this work focused on the isolation of *L. monocytgenes* from diseased sheep and biochemical characterization of the recovered isolates using traditional and Microbact™ 12L listeria identification systems.

Keywords: *Listeria monocytogenes*, Sheep, Phenotypic Identification, Biochemical Tests.

Introduction

Many dairy and poultry products contain many pathogens such as *Staphylococcus*, *E.coli* , *Salmonella*, and *Listeria monocytogenes* ***(Algammal et al., 2020a ; Algammal et al.,2020b)***. *L. monocytogenes*, a food-borne bacterium. It has now been found in many mammals and non-mammals, including agricultural ruminants ***(Low and Donachie, 1997).*** Their

toxins are released into the environment, leading to a wide range of diseases in humans and animals ***(Rawool et al., 2007 and Headley et al., 2014).*** Meningitis, neonatal losses, sepsis, and febrile gastroenteritis are invasive diseases that *L. monocytogenes* can cause in humans and farm animals ***(Schlech, 2000).*** Severe septicemia, brain inflammation, meningitis, meningo- and rhino-encephalitis, neonatal infections and gastroenteritis are also symptoms of listeriosis ***(Limmahakhun and Chayakulkeeree, 2013; Mateus and Lecuit, 2013 and OIE, 2014).*** Most animal infections are subclinical, but they can occasionally be life-threatening ***(OIE, 2014).*** Septicemia, encephalitis, and abortion types of listeriosis have all been observed in animals. The encephalitis, mastitis, rebreeding, and endometriosis of animals are all caused by *Listeria* ***(Malik et al., 2002).*** In sheep, it causes encephalitis and miscarriage and is mostly a disease of ruminants. Numerous food-borne outbreaks due to listeriosis have occurred across nations and continents since *L. monocytogenes* was first linked to humans a decade ago ***(Osman et al., 2020).*** More than 90 % of human listeriosis cases can be traced back to infected food ***(Mead et al., 1999).*** The ability of *L. monocytogenes* to survive in a variety of environments is owing to its success as a food-borne pathogen. As a result, its inclusion in food could pose a major health risk. Many food-borne illness outbreaks have been attributed to this psychrotrophic bacterium, which has the greatest hospitalization and mortality rates ***(Farber and Peterkin, 1991).*** Therefore, this work focused on isolating *L. monocytgenes* from diseased sheep and biochemical characterization of the recovered isolates using a traditional and Microbact™ 12L listeria identification system.

Materials and methods

1. Isolation of *Listeria monocytogenes*

This study was performed on diseased sheep suspected of being infected with listeriosis and suffering from nervous manifestation, septicemia, abortion, and mastitis. A total of 240 samples were collected aseptically from diseased sheep. The samples were incubated in Half Fraser broth (Oxoid, UK), and the tubes were incubated aerobically at 30±1 °C for 24±3 h. Subsequently, 0.1 ml of the enrichment broth was transferred to a new tube containing 10 ml of Fraser's secondary broth (Oxoid, UK) and the tubes were incubated at 35ºC for 24-48 h. Subsequently, 0.1 mL from the broth tubes showing darkening discoloration were streaked onto Oxford agar (Oxoid, UK) plates. The plates were incubated at 35±1ºC for 48 h and examined to check for listeria-

like colonies. Colonies were picked up and streaked onto TSYEA (Tryptone Soya Yeast Extract Agar) (Oxoid, UK) for purification of the isolates. The plates were incubated at 35ºC for 48 h. The purified isolates were further identified as previously described **(Markey *et al.*, 2013).**

2. Identification of *Listeria monocytogenes*

2.1. Detection of hemolysis

Heavy purified colonies from incubated TSYEA agar were inoculated on 5% sheep blood agar, and the plates were incubated at 35°C for 24-48 h to detect hemolysis. *L. monocytogenes* is β-hemolytic with bright light (a narrow clear) zone around the colonies ***(Yadav et al., 2010)***

2.2. Motility test

A sterile needle was used to pick a well-isolated colony and stab it onto the semisolid nutrient agar medium within 1cm of the bottom of the tube. The needle must be in the same line as it was removed from the medium. The tubes were incubated for 48 h at 25ºC and then examined for growth around the stab. *Listeria* species are motile with a typical umbrella-like growth **(MacFaddin, 2000)**.

2.3. Morphological characters

Smears from suspected *Listeria* pure colonies (16 to 24 h) were stained with Gram's stain and examined microscopically to observe their morphological characters.

2.4. Biochemical identification of *L. monocytogenes*

The purified listeria isolates were examined by different biochemical tests ***(Hitchins, 2001; Markey et al., 2013)*** as follows:

A. Catalase production test

On a clean glass slide, a drop of 3% hydrogen peroxide was placed. On the slide, a loopful of the bacterial colony was emulsified with a hydrogen peroxide solution. The formation of bubbles was identified as a positive reaction.

B. Carbohydrate fermentation

Heavy purified colonies on TSYEA were inoculated into 10 mL of purple cresol broth base containing sugars at 1%; L-rhamnose, glucose, sucrose, fructose, lactose, galactose, xylose, maltose and dextrose. Then, the tubes were incubated at 35 ºC for 24-48 h and evaluated for color changes. *Listeria* species produce acid with no gas (changes in color to yellow). *L. monocytogenes* doesn't utilize xylose.

C. Methyl red test

The methyl red Voges-Proskauer (MR-VP) broth medium was inoculated with a pure culture of the tested organism and incubated at 37 ºC for 48 h. After incubation, 5 drops of methyl red reagent were added immediately to the tube. The red colour represents a positive outcome.

D. Voges-Proskauer test

The isolated pure bacteria were inoculated into 5 mL of MR-VP

broth medium and incubated at 37 °C for 48 h. One mL of 40% potassium hydroxide solution, followed by 3 mL of alpha-naphthol solution, was added, and the mixture was thoroughly mixed. After 15 minutes, a bright pink colour indicates a positive reaction.

E. Urease test

Christensen's urea agar medium was prepared, autoclaved, and sterile urea solution (40%) was added at a temperature of 55 ºC. The bacteria were inoculated heavily on the surface of the agar medium's slope without stabbing. The inoculated tubes were incubated for 24 h at 37 ºC. The purple-pink to crimson tint development due to ammonia splitting signaled urea hydrolysis.

F. Oxidase test

Few drops of 1%NNN'N'-tetramethyl- phenylenediamine dihydrochloride solution were added to a sterile filter paper. A glass rode containing bacterial colonies was smeared into the reagent on the filter paper. A positive reaction is indicated by the rapid development of a deep blue colour at the site of injection.

G. Esculin test

The suspected organisms were inoculated into tubes containing 5 mL of esculin broth and incubated at 37 ºC for 48 h. The black colour indicates positive results.

H. Nitrate reduction test

Purified listeria culture was used to inoculate nitrate broth tubes. The tubes were incubated at 35 °C for 5 days before adding 0.2 mL of test reagents. A red-violet colour suggests the presence of nitrite as a result of nitrate reduction. If no colour was formed, powdered zinc was added to the tubes and they were left for 1 h. A red-violet color suggests that the nitrate was still present and had not been diminished.

I. Gelatin hydrolysis test

A heavy inoculum of test bacteria (18 to 24 h old) was inoculated by stabbing the nutrient gelatin medium tube 4-5 times (a half-inch). For 48 h, the inoculated tube was incubated with an uninoculated medium at 35°C or the optimal growth temperature of the test bacterium. The tubes were gently tilted, withdrawn from the incubator daily and placed in an ice bath in a refrigerator (4°C) for 30 minutes or until the control tube solidified to demonstrate that the liquefaction was caused by gelatinase activity as gelatin generally liquefies at 28°C and above. The tubes of the test organism were gently checked for liquefaction after 30 minutes of refrigeration. Negative test tubes were incubated for up to 2 weeks and then examined at regular intervals. Partial or total liquefaction of the inoculated tubes indicates positive results as the uninoculated control medium must be completely solidified after refrigeration (4°C).

J. Indole production test

This test demonstrates the ability of certain bacteria to decompose the amino acid tryptophan into indole, which accumulates in the medium. The test organisms were cultured in 3 mL of peptone water containing tryptophan at 37ºC for 24 h. A colorimetric reaction then tests indole. After incubation, 0.5 ml of Kovac's reagent was added to the inoculated tubes and the tubes were shaken gently. A red-colored ring indicates a positive result. Listeria isolates show negative results (yellow-brown ring).

K. Citrate utilization test

It was used to test the ability of the microorganism to utilize citrate as the sole carbon and energy source for growth and ammonium salt as the sole source of nitrogen. It was applied by making a single streak from the purified culture at the surface of Simmon's citrate agar slope and the tubes were then incubated at 37 ˚C for 24-48 h. Development of blue color indicates citrate utilization, while a negative result was detected by no change in the colour (remains green).

L. Reactions on TSI agar medium

Fermentation ability and hydrogen sulfide production of the bacterial isolates were tested by stabbing the suspected pure colonies on the bottom of the butt of TSI agar medium and then streaking in a zigzag manner over the slanted surface. The stabbed tubes were incubated at 37 ºC for 24-48 h. Carbohydrate fermentation is indicated by gas production and through the change in the color of the pH indicator from red to yellow. Detection of hydrogen sulfide production is indicated by blackening in the butt of the tube.

2.5. Confirmatory biochemical identification of *Listeria monocytogenes* using Microbact™ 12L listeria identification system (Oxoid, UK)

Each Microbact strip has 12 tests (11 sugar utilization tests plus a rapid hemolysis test). A single isolated colony from 18-24 h of *Listeria monocytogenes* culture was chosen, emulsified in suspending medium, and then completely mixed. Four drops of bacterial suspension were pipetted into each well using a sterile Pasteur pipette. One drop of the inoculum was placed on a non-selective medium for 24 h to assess purity. Preparation of positive and negative biological reaction colors (blue, yellow, black, pink and green) was done. Coloured liquids were poured into Microbact strip wells to match each positive or negative biochemical reactions. Depending on the inoculum, the inoculated strips were incubated for 4 or 18-24 h at 35 °C. In the sugar utilization test, reactions are shown by a color change and in the hemolysis test, sheep red blood cells are lysed. All test findings

were reported and the results were compared to the data table's recommendations to interpret the results. The Microbact™ Computer-Aided Identification Package was used to evaluate the results after incubation.

Results

1. Prevalence of *L. monocytogenes*

Thirty-one Listeria isolates out of 240 samples were recovered from diseased sheep with a prevalence rate of 12.9%.

2. Phenotypic identification of the recovered *L. monocytogenes*

2.1. Colonial appearance

The recovered isolates in this study exhibited black colonies with dimpled centers onto Oxford agar .

2.2. Detection of hemolysis on blood agar

All the recovered isolates showed a narrow zone of β hemolysis in the form of translucent greyish colonies with luxuriant or shiny grey smooth colonies.

2.3. Detection of motility on semisolid agar medium

All listeria isolates were motile and showed an umbrella pattern of motility.

2.4. Morphological characteristics

Films prepared from pure colonies of isolated *Listeria* isolates showed Gram-positive, non-sporulated and non-capsulated short rods. They were distributed individually, in short chains and sometimes in the form of V and Y letters **(Figure 1)**.

2.5. Biochemical identification of *L. monocytogenes*

Biochemical characterization tests used for presumptive identification of listeria isolates are shown in **Table 1**.

2.6. Confirmatory biochemical identification of *L. monocytogenes* isolates using Microbact™ 12L listeria identification system

The isolates were fully identified using the Microbact™ 12L listeria identification system, as shown in **Figure 2**.

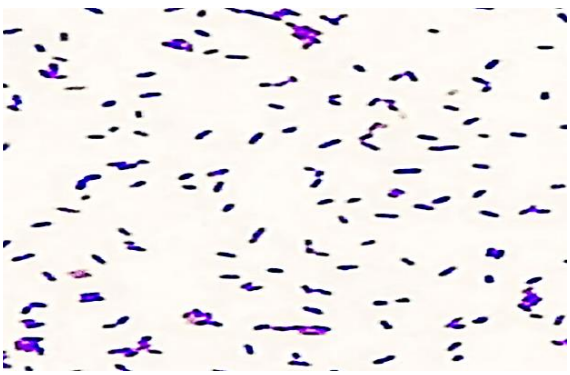

Figure 1: Morphological characters of *Listeria* isolates with Gram's stain

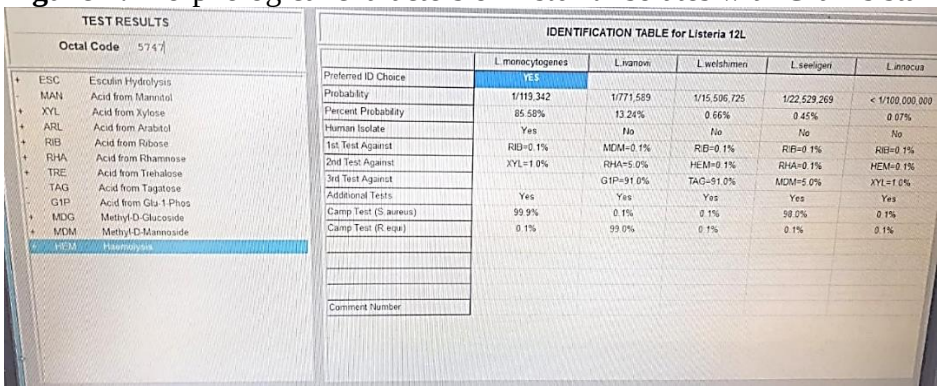

Figure 2: Identification of *L. monocytogenes* isolates using the Microbact™ 12L *Listeria* identification system.

Table (1): *Biochemical characterization tests of Listeria monocytogenes*

Biochemical test	Result
Catalase	+
Oxidase	-
Esculin hydrolysis	+
Urease	-
Nitrate reduction	-
Methyl red	+
Voges- Proskauer	+
Indole	-
H_2S production	-
Fermentation of sugar	
D-xylose	-
L-rhamnose	+
D-mannitol	-
Glucose	+
Sucrose	+
Fructose	+
Lactose	+
Galactose	+
Dextrose	+
Mellibiose	-
Maltose	+

+ Positive ,- Negative

Discussion

Listeriosis cases have been reported in several countries, especially Egypt, with many outbreaks ***(Kamar et al., 2014).*** As one of the most recently emerging zoonotic diseases, listeriosis is linked to the consumption of food and food

products that have been contaminated. Therefore, *L. monocytogenes* is of public health significance due to the frequent contamination of food products ***(Taha and Ahmed, 2017).*** Moreover, subclinical mastitis caused by *L. monocytogenes* is one of the essential features of bovine ***(Bourry et al., 1996)*** and ovine ***(Fthenakis et al., 1998)*** listeria mastitis. Therefore, the current study aimed to identify the isolated *L. monocytogenes* from diseased sheep via conventional microbiological techniques. *L. monocytogenes* are Gram-positive rods that are both aerobic and facultatively anaerobic, catalase-positive (although catalase-negative *Listeria* has been reported), oxidase-negative, fermentative in carbohydrates and generating acid without gas. Most strains are motile at 28 °C and non-motile at 37 °C ***Welshimer (1981).*** Traditional biochemical testing, which is time-consuming and takes a week to differentiate species using sugar utilisation assays, is a widely used alternative. *Listeria* tests like API *Listeria* (bio-Merieux, Marcy-Etoile France) and Micro-ID™ (Remel USA) have been verified extensively and are now part of standard techniques ***Cox (1997).*** The addition of esculin to the medium resulted in the production of colonies ranging from grayish-green to black, and in some cases, black media. Oxford agar ***(Curtis et al., 1989)*** has incorporated these components, but a medium that can discriminate between *L. monocytogenes* and other non-pathogenic *Listeria* spp. appears to be still needed, such as the ALOA medium ***(Ottaviani et al. 1997).*** However, Oxford agar has the advantage of significantly reducing the growth of contaminating microorganisms and providing a substantially less fastidious reading ***(Art and Andre, 1991)*** but cannot differentiate between *L. monocytogenes* and other *Listeria* spp***. (park et al.,2014).*** Herein, 31 (12.9%) listeria isolates were recovered from 240 collected samples giving bluish-green colonies on oxford medium. This prevalence rate was relatively similar to those reported in prior studies in Brazil 16.7%, ***(Monteiro et al., 2013),*** Iran 20.3%, ***(Haj Hosseini et al., 2014)*** and Ghana 12.2%, ***(Kwarteng and Wuni, 2018).*** There may be many reasons for this variation in the results, including differences in diet type, specimen counts, and geographic location ***(Nayak et al., 2015).***

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الملخص العربي

الليستريات هي ثالث أكثر الأمراض المنقولة بالغذاء شيوعًا وتعتبر واحدة من أخطر أنواع العدوى البكتيرية حيوانية المصدر على مستوى العالم. يهدف هذا البحث إلى عزل وتوصيف (*L. monocytogenes*) من الأغنام المريضة في مصر. تم إخضاع ما مجموعه 240 عينة جمعت معقمًا من الأغنام لعزل وتوصيف كيميائي حيوي لـ *L. monocytogenes*. تم عزل 31 من الليستريات (12.9٪) من الضأن المريضة. كانت جميع العزلات المختبرة موجبة لاختبارات الكاتلا، وفوجس-بروسكار ، والتخمير الكربوهيدرات ، والإسكولين ، والتحلل المائي للجيلاتين، واختبارات الميثيل الأحمر. وفي الوقت نفسه، كانت سلبية بالنسبة لاختبارات اختزال الأوكسيداز، والإندول، واليورياز، والنترات ولم يستخدموا السترات في اختبار استخدام السترات. أنتجت عزلات الليستريا بعقب أصفر / مائل أصفر على وسط أجار TSI بدون إنتاج غاز H2S أو غاز.