DETECTION OF VIRULENCE ASSOCIATED GENES IN
LISTERIA MONOCYTÖGENES ISOLATED FROM
DISEASED FARM ANIMALS

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ABSTRACT

A total of 631 different samples (milk, faeces, brain and Cerebrospinal fluid) were collected from 268 diseased farm animals and emergency slaughtered animals (80 sheep, 60 goat, 75 cattle and 53 buffaloes) from Dakahlia Governorate in 2014 and 2015. The samples were tested for presence of L. monocytogenes by isolation and biochemical identification.

The results revealed that L. monocytogenes was presented in diseased and emergency slaughtered sheep, goat, cattle and buffaloes in ratio of (36.5%), (28.67%), (24.48%), (17.4%) respectively. Using multiplex PCR technique, detected the presence of virulence-associated genes
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INTRODUCTION

*L. monocytogenes* is a food borne pathogen of major concern with regard to public and animal health. This bacterium affects a wide range of mammalian species, most commonly humans and domestic ruminants (*Chen et al., 2007*). *L. monocytogenes* isolates were small, Gram-positive rods, negative for oxidase and urease, and test-positive for methyl red and Voges–Proskauer, had the ability to grow at 35°C, were catalase-positive and motile in wet mounts. They utilized dextrose, esculin and utilized rhaminose with production of acid but failed to utilize xylose *Osman et al., (2014)*. Clinical manifestations are similar in all susceptible hosts and include septicemia, abortion, severe gastroenteritis, and central nervous system (CNS) infections, such as meningitis, meningoencephalitis, and rhombencephalitis *Zhang et al., (2003)*. Identification of infected animals was necessary due to the likely causal link to several outbreaks of listeriosis. *Rawool et al., (2007)*.

Four key of *L. monocytogenes* virulence genes (i.e plcA, hly, actA, and plcB) that are critical for the intracellular life cycle *Ward et al., (2004)*. The hemolysin (listeriolysin O), two distinct phospholipases, a protein (ActA), several internalins, and others had been identified and extensively characterized at the molecular and cell biologic levels *Kathariou S (2002)*.

This study was done to throw spot light on molecular characterization of *L. monocytogenes* infection in some farm animals including sheep, goat, cattle and buffaloes in El-Dakahlia governorate.
The namely *inlA, inlC, inlJ, plcA, actA, hlyA* and *jap, inlB* and *plcB* genes for *L. monocytogenes* were chosen as target genes throughout this study.

**MATERIAL AND METHODS**

A total of 631 samples were collected from 268 diseased farm animals and emergency slaughtered animals (80 sheep, 60 goat, 75 cattle and 53 buffalo). Such samples including faeces (80), cerebrospinal fluid (17), milk (32) and brain (60) from diseased sheep, faeces (60), cerebrospinal fluid (11), milk (17) and brain (48)] from diseased goat, faeces (75), milk (62) and brain (46) from diseased cattle and faeces (53), milk (41) and brain (29) from diseased buffaloes.

These samples were collected from different village of Dakahlia Governorate. Samples were collected separately in sterile plastic bag, well identified and transported in ice box (4°C) under strict hygienic condition to Mansoura Veterinary laboratory within 2 hrs.

**Isolation of L. Monocytogenes:**

The technique recommended by United Stated Department of Agriculture (USDA), Food Safety and Inspection Service "FSIS" (1989) and *FAO (1992)* was adapted.

Twenty five gm. from each brain- faeces sample were aseptically weighted and homogenized in primary selective *Listeria* Enrichment Broth, University of Vermont Medium provided from Biolife (LEBUVM\textsubscript{I}) and incubated at 30°C for 24 hours then 0.1 ml of incubated (LEBUVM\textsubscript{I}) will be transferred to 10 ml (LEBUVM\textsubscript{II}) and incubated at 33- 37°C after 24 hours of incubation a loopful from enrichment culture UVM\textsubscript{II} broth were streaked onto PALCAM agar plates (oxoid CM,877).
containing selective supplement (oxoid SR150), then incubated at 35-37°C for 24-48 hours (*Jemmi and Keusch, 1994*).

Milk samples and C.S.F.: (*FDA, Lovett et.al., 1987*): twenty five ml. of milk were added to 225ml. of Enrichment Broth Modified tryptone soya broth containing 0.6% yeast extract, Nalidixic cid 40mg/l, Acriflavine hydrochloride 15mg/l and incubated at 30°C for 48 h then 0.1 ml of enrichment broth was streaked on PALCAM agar plates (oxoid CM,877) containing selective supplement (oxoid SR150), then incubated at 30°C for 48 hours.

**Identification of *L. Monocytogenes (ISO, 11290)*:**

The suspected colonies (drop-like, black with brown hallow with sunken center) were picked up and subcultured on Trypticase Soya agar supplemented with 0.6%-yeast extract (TSA-YE) and incubated at 30°C for 24 then identified according to *FAO (1992)*, Bacterial films from the suspected pure colonies were stained with Gram's stain and using appropriate biochemical tests (Catalase test, H₂S production, D-glucose, salacin fermentation (Purple Agar Base, Oxidase test, and Vogas proskouer reaction and umbrella type motility at 22°C. Further identification to species were done using heamolysis on blood agar, Nitrate reduction, acid production from D-xylose, L-raminose, D-Mannitol and methyl- d- mannoside.

**Detection of associated virulence genes of 10 *L. monocytogenes isolates by multiplex PCR for :**

Bacterial strans :A collection of 10 *L.monocytogenes* were investigated. The collection contained (5,2, 1 and 2) straines from brain, C.S.F., faeces and milk respetivelly from ruminant with listeric encephalitis.
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Template DNA extraction according to (Liu et. al., 2007).

AmpliTaq Gold® 360 Master Mix (U.S.A., Applied Bio System Code No.439881) and Oligonucleotides primer

**Table (1):** Oligonucleotides primer sequences and expected product sizes of the multiplex PCR:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5'→3')</th>
<th>Expected product size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>inlA</td>
<td>Forward: ACGGTAACGGGACAAATGC</td>
<td>800 bp</td>
<td>Liu et. al., 2007</td>
</tr>
<tr>
<td></td>
<td>Reverse: CCCGACAGTGGTGCTGATT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>inlC</td>
<td>Forward: AATTCCCCACAGGACACAACC</td>
<td>517 bp</td>
<td>Sanghun et. al., 2012</td>
</tr>
<tr>
<td></td>
<td>Reverse: CGGGAATGCAATTTCACCTATA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>inlJ</td>
<td>Forward: TGTACCCCCGCTTACACAGTT</td>
<td>238 bp</td>
<td>S. Kaur et. al., 2007</td>
</tr>
<tr>
<td></td>
<td>Reverse: AGCGGCTTGGCAGTCTAAATA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>inlB</td>
<td>Forward: AAAGCAGGATTTCATGGGAG</td>
<td>146 bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse: ACATAGCCTTGTTTGGTCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>plcB</td>
<td>Forward: GGGAAATTTGACACACGTTT</td>
<td>261bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse: ATTTTCGGGTAGTCCGCTTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>plcA</td>
<td>Forward: CTGCTTGAGCGTTTCATGTCTCATCCCCC</td>
<td>1484 bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse: CATGGGTTTCACCTCTCTTCTAC</td>
<td></td>
<td>S. Kaur et. al., 2007</td>
</tr>
<tr>
<td>hly A</td>
<td>Forward: GCAGTTGCAAGCGCTTGGGATGAA</td>
<td>456 bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse: GCAACGTATCTCCAGAGTATCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>jap</td>
<td>Forward: ACAAGCTGCACCTGTTGACGTTAAG</td>
<td>131bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse: TGCAACGCTGTGTTAGTAGCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>act A</td>
<td>Forward: CGCCGCAGAATTTAAAAAGA</td>
<td>839bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse: ACGAGGAACCAGGCTGCTG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The mixture used and the reaction conditions for Multiplex PCR for the detection of **inlA**, **inlC** and **inlJ** were according to Liu et. al., (2007).

The mixture used and the reaction conditions for Multiplex PCR for the detection of **inlB**, **plcB** were according to Sanghun et. al., (2012).
The mixture used and the reaction conditions for Multiplex PCR for the detection of plcA, hylA, jap and actA were according to S. Kaur et al., (2007)

Agarose gel electrophoresis was done according to Sambrook et al., (1989).

RESULTS

Total incidence of *Listeria monocytogenes* in diseased farm animals samples by culture isolation method:

It is evident from the table (2) that the prevalence of *L. monocytogenes* in diseased sheep was (36.5) the higher rate of isolation was from C.S.F (47.05%) followed by brain (43.3%), milk (32.5%) and from feaces (28.1%). The prevalence of *L. monocytogenes* in diseased goat was (28.67) isolated from (23.3%), (29.4%), (31.25%) and (36.36%) from feaces, milk, brain, and C.S.F respectively, while in diseased cattle the prevalence of *L. monocytogenes* was (24.48) from faeces (28%), milk (20.9%) and brain (24.48%). As shown in table (2) that the prevalence of *L. monocytogenes* in diseased buffaloes isolated in a ratio of (26.4%), (12.1), and (10.3%) from faeces, milk and brain respectively.

**Table (2):** Total incidence of *L. monocytogenes* in diseased farm animals samples by culture isolation method:

<table>
<thead>
<tr>
<th>Animals</th>
<th>No. of animals</th>
<th>Positive</th>
<th>Faeces</th>
<th>Milk</th>
<th>Brain</th>
<th>C.S.F</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>C.S.F</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Faeces</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

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Multiplex Polymerase chain reaction (PCR) for detection of associated virulence genes of *L. monocytogenes*:

The multiplex PCR allowed amplification of 9 virulence-associated genes of *L. monocytogenes*, namely *plcA*, *actA*, *hlyA* and *jap*, *inlB*, *plcB*, *inlA*, *inlC* and *inlJ*. to their respective base pairs, 1484, 839, 456, 131, 146, 261, 800, 517 and 238 bp PCR products, respectively, each represented by a single band in the corresponding region of the DNA marker ladder, all of the amplification products were of the expected size, except for the *actA* gene did not produced by ay examined strain in this study.

**Table (3):** Detection of virulence associated genes in 10 *L. monocytogenes* isolates by multiplex PCR:

<table>
<thead>
<tr>
<th>No. of the isolates</th>
<th>Virulence associated genes</th>
<th>Animal species</th>
<th>Origin of the sample</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>inlA</em></td>
<td><em>inlC</em></td>
<td><em>inJ</em></td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Sheep: Feaces

cattle: Milk

Sheep: C.S.F

cattle: Brain

Sheep: Mil

Sheep: Brain

Sheep: C.S.F

goat: Brain

cattle: Brain
Detection of virulence associated genes (*inlA* (50pmol), *inlC* (30pmol) and *inlJ* (25pmol)) in 10 *L. monocytogenes* isolates by multiplex PCR:

**Photo (1)** showed that *L. monocytogenes* isolates produced (60%), (80%) and (80%) of *InlA*, *InlC* and *inlJ* gene respectively.

**Photo (1):** Ethidium bromide stained 2% agarose gel electrophoresis showing the standard PCR of the 1-10: suspected *L. monocytogenes* DNA from the examined samples +ve: positive control of *L. monocytogenes*, ve: negative.

Detection of virulence associated genes (*plcB* and *inlB*) in 10 *L. monocytogenes* isolates by multiplex PCR:

The results in **Photo (2)** show that (70%) of *L. monocytogenes* isolates produced *plcB* gene and (30%) of *L. monocytogenes* isolates produced *inlB* gene.
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Photo (2): Ethidium bromide stained 2% agarose gel electrophoresis showing the standard PCR of the 1-10: suspected *L. monocytogenes* DNA from the examined samples +ve: positive control of *L. monocytogenes* and -ve: negative.

Detection of virulence associated genes (*jap*, *hylA*, *actA* and *plcA*) 50pmol in 10 *L. monocytogenes* isolates by multiplex PCR:

It is evident from photo (3) that (90%) of *L. monocytogenes* isolates produced *hylA* gene, (80%) of *L. monocytogenes* isolates produced *jap* gene and (50%) of *L. monocytogenes* isolates produced *plcA* gene.

Photo (3): Ethidium bromide stained 2% agarose gel electrophoresis showing the standard PCR of the 1-10: suspected *L. monocytogenes* DNA.
from the examined samples +ve: positive control of *L. monocytogenes* and -ve: negative.

**DISCUSSION**

Enrichment of *L. monocytogenes* was done on the UVM\textsubscript{I} and UVM\textsubscript{II} broth *(Jemmi and Keusch 1994)*. Isolation of *L. monocytogenes* were done on the PALCAM media with selective supplement, typical colonies recovered were grey-green 1.5- 5 in diameter, and have black sunken centers due to esculin hydrolysis as the results obtained by *(Parihar et. al., 2008)*.

Results in table (2) for the bacteriological examination on 268 diseased farm animals and emergency slaughtered animals (80 sheep, 60 goat, 75 cattle and 53 buffalo) revealed that (36.5%, 28.6%, 24.48% and 17.4 %) isolated from sheep, goat, cattle and buffaloes respectively were nearly similar to the results that recorded by *(Simranpreet et. al., (2010); Antônio et. al., 2014; Osman et. al., 2014)*. Higher figures were reported by *(Esteban et. al., (2009)*. Meanwhile lower prevalence was recorded by *(F. A. Lawan et. al., (2013)*.

The variation in the isolation rate may be due to variation in methods of isolation *(Jemmi and Keusch, (1994)* and variation in animal husbandry practice and type of animal species *(F. A. Lawan et. al., (2013)*.

Results in Table (2) showed that *L. monocytogenes* was recovered from the C.S.F., brain, faeces and milk of the examined sheep was (47.05%), (43.3 %), (32.5%), and (28.1%) respectively. These results agree with *(Elgamal A. M. 2003; Antônio et. al., 2014)*.
The results achieved of the isolation of *L. monocytogenes* from fecal samples of sheep agree with *F. A. Lawan et al., (2013)*. Lower rate of isolation were recognized by *Esteban et. al., (2009)*.

The isolation of *L. monocytogenes* from sheep milk samples agree with *Osman et al., (2014)*. Lower rate of prevalence recorded by *Ebrahim et. al., (2014)*.

**Table (2)** declares that that *L. monocytogenes* was recovered (36.36%), (31.25 %), (23.3%) and (29.4%) from the C.S.F., brain, feaces and milk of the examined goats.

The isolation rates of *L. monocytogenes* from C.S.F. and brain samples of diseased goat were in the ranges which agree with *Antônio et. al., (2014)*. Lower rate of isolation were recognized by *Shivasharanappa et. al., (2014)*.

The result of isolation of *L. monocytogenes* from milk of diseased goat samples agrees with *Osman et. al., (2014)*. The lower rate recognized by *Ebrahim et. al., (2014)*.

The isolation of *L. monocytogenes* from faeces of diseased goat samples were in the range which agrees with *F. A. Lawan et. al., (2013); Farad et. al., (2013)*

*L. monocytogenes* were recovered from the brain, faeces and milk of the examined cattle in a ratio of (24.48%), (28%) and (20.9%) respectively as shown in **Table (2)**; which agree with *Antônio et. al., (2014)*.
The isolation of *L. monocytogenes* from fecal samples of diseased cattle agrees with *F. A. Lawan et. al., (2013)*.

Isolation of *L. monocytogenes* from milk samples of diseased cattle agrees with *Alejandra et. al., (2009)*; while higher rate of isolation of *L. monocytogenes* recorded by *Deepansh et. al., (2012)*.

**Table (2)** approved that *L. monocytogenes* recovery rate from the brain, feaces and milk of the examined buffaloes were (10.3 %), (26.4%), (12.1%) respectively, these results agree with *Simranpreet et. al., (2010)*. Lower rate of isolation was recorded by *Chaudharia et. al., (2004)*.

The isolation of *L. monocytogenes* from milk samples of diseased buffaloes agrees with *Deepansh et. al., (2012); Farad et. al., (2013)*.

Multiplex PCR assay was carried out for detection of *L. monocytogenes* and presence of *inLA, inLC, inlJ, inlB, plcB, plcA, hlyA, actA and jap* genes.

However, some *L. monocytogenes* strain may lack one or more virulence determinants because of some mutation *Cooray et. al., (1994)*.

As shown in photo (1) *L. monocytogenes* isolates produced (60%), (80%) and (80%) of *InlA, InlC* and *inlJ* gene respectively. The combined application of *inLA* which is species-specific, *inLC* and *inLJ* gene primers in a multiplex PCR confirm *L. monocytogenes* species identity and its potential virulence *Jaradat et. al., (2002)*.

The results in photo (2) show that (70%) of *L. monocytogenes* isolates produced *plcB* gene and (30%) of *L. monocytogenes* isolates...
produced \textit{inlB} gene. \textit{InlB} is not an important virulence factor for listeriosis \textit{Liu et. al., (2007)} recognized that \textit{InlB} gene failed to be recognized with serotype 4 strains.

The result in photo (3) that (90\%) of \textit{L. monocytogenes} isolates produced \textit{hylA} gene, (80\%) of \textit{L. monocytogenes} isolates produced \textit{jap} gene and (50\%) of \textit{L. monocytogenes} isolates produced \textit{plcA} gene.

\textit{Roche et. al., (2009)} stated that some isolates of \textit{L. monocytogenes} harbor \textit{inlA} gene with or without \textit{hlyA} gene which supported the usefulness of studying the pathogenic potential of strains.

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Kشف الجينات المتعددة المرتبطة بالضراوة في ميكروب الليستيريا مونوسيتوجين المعزولة من حيوانات المزرعة المريضة.

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معهد بحوث صحة الحيوان، معهد المنصورة الفرعى.
Detection Of Virulence Associated Genes In ...


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من الفحص البكتيري لعدد 631 عينة (لب، براز، مخ و سائل النخاع الشوكي) تم تجميعها من 80 أغنم، 60 ماعز، 75 أبقار و 53 جاموس) من الحالات المريضة وحالات الرباط الاضطراري في الفترة (2014-2015) من أجل عزل ميكروب ليستيريا هيدنسيتوجين وتاقة النسبة الاجابة في الأبقار (24.48)، الجاموس (17.4%)، الأغنام (36.5%) و الماعز (28.67%). بدء تفاعل البلمرة المتسلسل وتفاعل البلمرة المتسلسل المركب للفترات المصنفة الليستيريا هيدنسيتوجين ظهرت أحجام القواعد النيتروجينية لجينات الاضرارة (inlJ, inlB, plcB, hylA, jap, inlA and inlC) في العينات المعزولة بنسب متزايدة.

actA plcA