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DETECTION OF VIRULENCE ASSOCIATED GENES IN *LISTERIA MONOCYTOGENES* 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, 60goat, 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

(inlA, inlC, inlJ, inlB, plcB, hylA, jap and plcA).

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 governerate.

The namely *inlA, inlC, inlJ. plc*A, *act*A, *hly*A and *jap, inlB* and *plcB* genes for *L. monocytogenes* were chosen as target genes throughout this study.

MATERIAL AND METHODS

A total of 631samples were collected from268 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^{o}C) under strict hygienic condition to Mansoura Veterinary laboratory within 2hrs.

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 ($LEBUVM_I$) and incubated at 30°C for 24 hours then 0.1 ml of incubated ($LEBUVM_1$) will be transferred to 10 ml ($LEBUVM_{II}$) and incubated at 33- 37°C after 24 hours of incubation a loopful from enrichment culture UVM_{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 24then 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, H_2S 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 respectivelly from ruminant with listeric encephalitis.

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:

Gene	Primer sequence (5'→3')		Expected product size (bp)	Reference
inlA	Forward	ACGAGTAACGGGACAAATGC	800 bp	***Liu et. al., 2007***
	Reverse	CCCGACAGTGGTGCTAGATT		
inlC	Forward	AATTCCCACAGGACACAACC	517 bp	
	Reverse	CGGGAATGCAATTTTTCACTA		
inlJ	Forward	TGTAACCCCGCTTACACAGTT	238 bp	
	Reverse	AGCGGCTTGGCAGTCTAATA		
inlB	Forward	AAAGCACGATTTCATGGGAG	146 bp	***Sanghun et. al., 2012***
	Reverse	ACATAGCCTTGTTTGGTCGG		
plcB	Forward	GGGAAATTTGACACAGCGTT	261bp	
	Reverse	ATTTTCGGGTAGTCCGCTTT		
plc A	Forward	CTGCTTGAGCGTTCATGTCTCATCCCCC	1484 bp	***S. Kaur et. al., 2007***
	Reverse	CATGGGTTTCACTCTCCTTCTAC		
hly A	Forward	GCAGTTGCAAGCGCTTGGAGTGAA	456 bp	
	Reverse	GCAACGTATCCTCCAGAGTGATCG		
jap	Forward	ACAAGCTGCACCTGTTGCAG	131bp	
	Reverse	TGACAGCGTGTGTAGTAGCA		
act A	Forward	CGCCGCGGAAATTAAAAAAAGA	839bp	
	Reverse	ACGAAGGAACCGGGCTGCTAG		

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:

Animals	No. of animals	Type and number of samples					Positive									
		Faeces	Milk	Brain	C.S.F	Total	Faeces		Milk		Brain		C.S.F.		Total	
							No.	%	No.	%	No	%	No	%	No	%

Sheep	80	80	32	60	17	189	26	32.5	9	28.1	26	43.3	8	47.05	69	36.5
Goat	60	60	17	48	11	136	14	23.3	5	29.4	15	31.25	4	36.36	39	28.67
Cattle	75	75	62	46	0	183	21	28	13	20.9	13	24.48	0	0	48	24.48
Buffaloe	53	53	41	29	0	123	14	26.4	5	12.1	3	10.3	0	0	24	17.4
Total	**268**	**268**	**152**	**183**	**28**	**631**	**75**	**27.98**	**37**	**20.55**	**47**	**25.68**	**12**	**42.85**	**180**	**27.31**

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 *plc*A, *act*A, *hly*A 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:

No. of the isolates	Virulance associated genes									Animal species	Origin of the sample
	inlA	***inlC***	***inlJ***	***inlB***	***,plcB***	***plc*A**	***act*A**	***hly*A**	***iap***		
1	-	-	-	-	-	-	-	-	-	Sheep	Feaces
2	-	-	-	-	-	-	-	+	+	cattle	Milk
3	+	+	+	-	+	-	-	+	+	Sheep	C.S.F
4	-	+	+	-	+	-	-	+	+	cattle	Brain
5	-	+	+	-	-	-	-	+	+	Sheep	Milk
6	+	+	+	+	+	+	-	+	+	Sheep	Brain
7	+	+	+	+	+	+	-	+	+	Sheep	C.S.F
8	+	+	+	+	+	+	-	+	+	goat	Brain
9	+	+	+	-	+	+	-	+	+	cattle	Brain

10	+	+	-	-	+	+	-	+	+	buffloe	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.

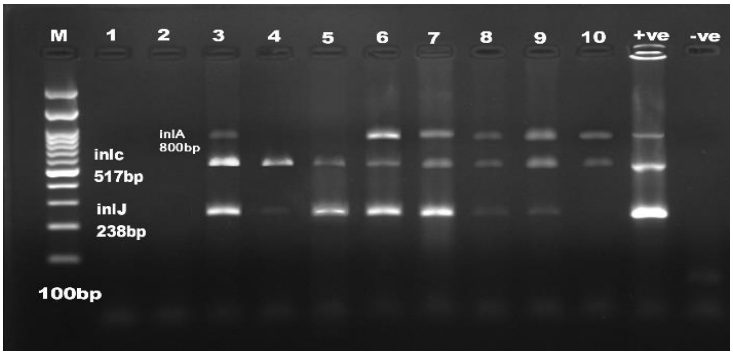

Photo (1): Ethidium bromide stained 2% agarose gel electrophoresis showing the standard PCR of the 1-10: suspected *L. monocytogenes* DNA from the examined samples +ve: positive control of *L. monocytogenes* , ve: negative.

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.

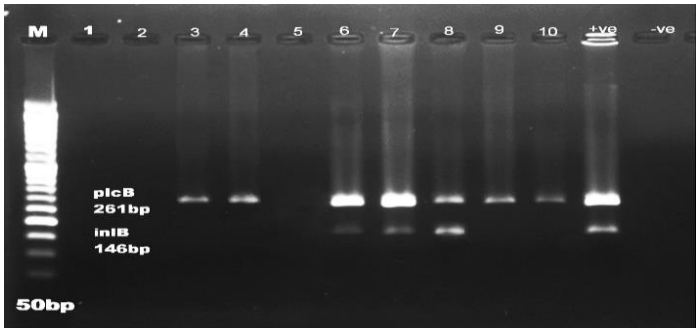

Photo (2): Ethidium bromide stained 2% agarose gel electrophoresis showing the standard PCR of the 1-10: suspected *L. monocytogenes* DNA from the examined samples +ve: positive control of *L. monocytogenes* and -ve: negative.

Detection of virulence associated genes (*jap, hylA, act*A and *plc*A) 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 *plc*A gene

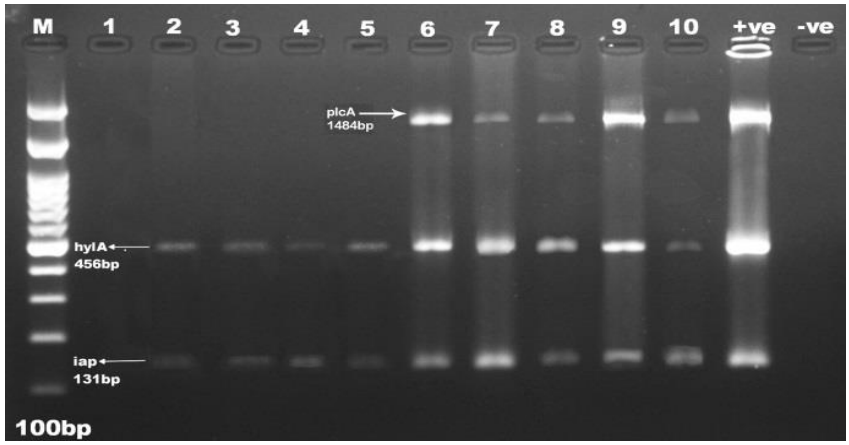

Photo (3): Ethidium bromide stained 2% agarose gel electrophoresis showing the standard PCR of the 1-10: suspected *L. monocytogenes* DNA

from the examined samples +ve: positive control of *L. monocytogenes* and -ve: negative.

DISCUSSION

Enrichment of *L. monocytogenes* was done on the UVM_I and UVM_{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 buffloes 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 *inlB* gene. *InlB* is not an important virulence factor for listeriosis ***Liu et. al., (2007)*** recognized that *InlB* gene failed to be recognized with serotype 4 strains.

The result in 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 *plc*A gene.

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

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كشف الجينات المتعددة المرتبطة بالضراوة فى ميكروب الليستيريا مونوسيتوجين المعزولة من حيوانات المزرعة المريضة.

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من الفحص البكتيري لعدد 631 عينة (لبن, براز, مخ و سائل النخاع الشوكى) تم تجميعها من (80 أغنام , 60 ماعز ,75 أبقار و 53 جاموس) من الحالات المريضة وحالات الذبح الاضطراري فى الفترة (2014–)2015 من أجل عزل ميكروب ليستيريا مونوسيتوجين وكانت النسبة الايجابية فى الأبقار (24.48%), الجاموس (17.4%), الأغنام (36.5%) و الماعز(28.67%). بإجراء تفاعل البلمرة المتسلسل و تفاعل البلمرة المتسلسل المركب للعترات المصنفة الليستيريا مونوسيتوجين ظهرت أحجام القواعد النيتروجينية لجينات الضراوة(*inlJ, inlB, plcB, hylA, jap, inlA* and *inlC* *actA plcA*) فى العترات المعزولة بنسب متفاوتة.