Molecular characterization of *Listeria monocytogenes* isolated from milk

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Abstract

A total of 560 various raw milk samples were collected from different dairy farms to examine the presence of *L.monocytogenes*. These isolates were further characterized by biochemical tests and Polymerase Chain Reaction (PCR). *L.monocytogenes* was isolated in 72 of the 560 samples (12.9%). Based on PCR, All the examined *L.monocytogenes* were positive for Listeriolysin O gene (*hly* A) and P60 (*iap*).The findings of this study show that consumption of raw milk could be a potential risk of human listeriosis.

Introduction

L.monocytogenes is ubiquitous as it has been reported in diverse environments including water, grass, silages, decomposing organic matter, soil and feces ***(Hassan et al, 2001).***The principal source of infection for ruminants is spoiled silage ***(Weidmann, 2003),*** although it may come from water or feed contaminated by avian or insect vectors ***(Cooper and Walker, 1998).***

L.monocytogenes is a Gram-positive bacteria, asporogenous, catalase positive, small size, with round extremities, first described in 1926 in Cambridge, United Kingdom ***(Aarnisalo et al, 2008).***It is a halo tolerant, facultative anaerobic, non-spore forming rod bacterium ***(Larson et al, 1999),*** it can grow in a wide pH range from 4.6 to 9.5 and can grow in low water activity environments as 0.90 ***(Gandhi and Chikindas, 2007).***

L.monocytogenes is an important food-borne pathogen and has been associated with outbreaks and sporadic cases of Listeriosis ***(Mead et al, 1999).***The important characteristics of *L.monocytogenes* contributing to food borne transmission are the ability to grow as low as -0.4°C, resist heat, salt, nitrite, acidity, withstand osmotic stress and survive mild preservation treatment measures commonly used to control the growth of organisms in food ***(Jalali and Abedi, 2007).***

Therefore, the aim of the present work was to determine the molecular characterization of *L.monocytogenes* isolated from milk to achieve that, the following steps was done:

1. Isolation and identification of *L.monocytogenes* from different

raw milk samples which collected from different dairy farms by different biochemical tests and Listeria Rapid Test**, *(FT0401, England).***

2.Application of PCR technique as a diagnostic tool for *L.monocytogenes* isolates by detection of two specific genes which are Listeriolysin O gene (*hly*A) and p60 gene (*iap*).

Material and methods

Samples: A total of 560 raw apparently healthy milk samples were collected aseptically from different Egyptian dairy farms. All samples were quickly transported to the laboratory of microbiology for examination.

Inoculums and samples preparation

The pre-enrichment media is **(Listeria oxford enrichment broth) (Oxford, CM862)**: in which the samples were enriched for 48 hr at 35 °C. Enriched broth was then plated on to **Listeria selective agar medium (Oxford, CM 856),** the plates were incubated for 48 hr at 37°C, the suspected colonies (smooth, round, black colonies) were inoculated then at 35°C for 24 hr on to Tryptic Soy Agar-Yeast Extract (TSA-YE). For purification, this step was repeated and repeating plating on oxford agar plates to obtaining pure colony used for identification include morphological examination (Microscopical examination, motility test and haemolytic activity), biochemical examination: (Oxidase, catalase and CAMP test) described by ***(Hitchins, 1995)*** and Oxoid Listeria Rapid Test, code: (FT0401, England).

Extraction of bactereial DNA:

Extraction of DNA from *L.monocytogenes* isolates by boiling method according to ***Bansal et al (1996).***

DNA amplification:

Two oligonucleotide primers were designed (Oligo Macrogen Og130527-015 seoul,153-781 Korea), according to the determined sequence of the *hlyA* gene (GenBank accession no. M24199) and the *iap* gene (GenBank accession no. AF532293).The sequences, designated as the species-specific PCR assay the primer pair consisting of primer (*iap* F and *iap* R) was used to amplify a 370 base pair (bp) region of the *iap* gene ***Kohler et al (1990)*** and (LM1F and LM2R) was used to amplify a 701 base pair (bp) region of the *hly*A gene ***Herman et al, (1995).***

Electrophoretic analysis

Was done according to ***(Sambrook & Russel, 2001).***

Results & discussion:

As seen in Table (1), out of 560 samples 112 were positive (20%) in milk, by culturing in (Oxford agar medium) with characteristic black, smooth coloured colonies with aesculin hydrolysis as which recorded by ***(Art and Andre, 1991** and **Emek et al, 2011).***

Table (2), out of 112 positive samples 72 were positive for *L.*

monocytogenes and the other infected by other organisms in (Oxford agar medium) depending on colonial morphology. Later these colonies were identified by different biochemical tests and Gram staining to differentiate between *L. monocytogenes* and other organisms as shown in Table (3)

Table(4), out of 560 samples 72 were positive for *L. monocytogenes*(12.9%) these results similar to that obtained by ***(Wagner et al, 2007)*** who detected *L. monocytogenes* in cheese processing and the prevalence was considerably higher as 50 of 181 (27.62%) samples were found positive in Austria, however, most of samples were from cheese. Also ***(Edward et al, 2011)*** reported that of samples tested (6.3%) of milk, (13.1%) of processing environment and (12.3%) of samples external to the processing environment were positive to *L.monocytogenes* but not similar to obtained by previous studies reported that *L. monocytogenes* was found between (1.25–7%) of the isolation rate in milk samples ***(Hàyes et al, 1986*** *and* ***Lovett et al., 1987)***.

Morphological characterization:

Gram staining of the suspected colonies showed Gram positive bacilli, rod shape with round extremities, small in size and arranged in short chains, sometimes in form of the letters V and Y As regards to motility test, the suspected *L. monocytogenes* colonies showed a surface umbrella shaped growth or tumbling motility. By the hemolytic activity of *L. monocytogenes*, the suspected colonies produce a slightly cleared zone of haemolysis. The same finding was obtained by ***(Capita et al, 2001; Abeer et al, 2012; Aarnisalo et al, 2008*** *and* ***Abbas and Jaber, 2012).***

Biochemical identification:

Table (5), *L. monocytogenes* showed positive result with catalse and CAMP test and negative result with oxidase test. These findings are in agreement with that illustrated by ***(Arun, 2008*** *and* ***Deepansh et al, 2012).***

Polymerase Chain Reaction (PCR) for *L. monocytogenes* isolates:

The results showed that 72 samples of 112 suspected samples were positive for *hly*A gene and *iap* gene on using its specific primers of *hly*A gene and *iap* gene. as shown in **Photo (1)** and **Photo (2).** As which recorded by, ***(Hossein et al, 2013),*** who examined 446 raw milk samples for the presence of Listeria species and characterized the isolates by biochemical tests and PCR. Listeria species were isolated in 83 out of the 446 milk samples (18.6%) and the highest prevalence was detected in cow raw milk samples (22.5%) followed by raw sheep milk samples (16.4%) and finally raw goat milk samples (4.9%) and the most common species isolated were *L. innocua* and *L. monocytogenes.*

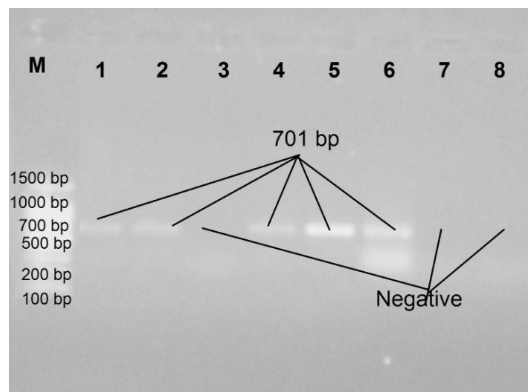

Photo (1)*: Agarose gel electrophoresis of amplified hlyA gene PCR product (701bp).*

M**:** DNA Marker, Lane 1, PCR positive control for *hly*A gene, Lane 2, 4, 5, 6: are PCR product
(+) from the *L. monocytogenes* isolated strains, Lane 3, 7: negative, Lane 8 negative control (DNA from *E.coli*).

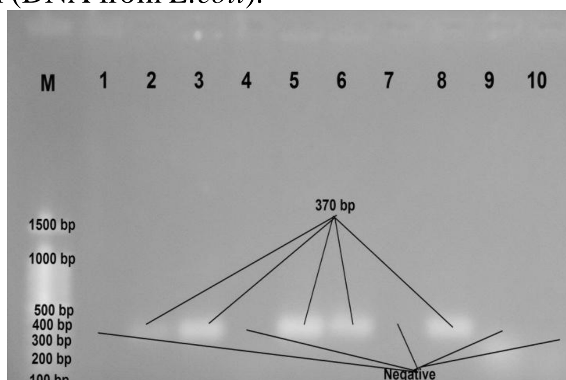

Photo (2):*Agarose gel electrophoresis of amplified iap gene PCR product (370bp).*

M**:** DNA Marker, Lane 1 negative control (DNA from *E.coli*), lane 2, PCR positive control for *iap* gene, Lane 3,5,6,8 are PCR product (+) from the *L. monocytogenes* isolated strains, Lane 4, 7, 9, 10 negative for *L.monocytogenes*.

Table (1): *An incidence of positive relative to negative (apparently normal) milk samples by culturing in specific medium of L. monocytogenes (Oxford agar medium).*

Total Examined Samples	Negative (apparently normal) samples		positive samples	
No.	No.	%	No.	%
560	448	80	112	20

Table (2): *An incidence of L. monocytogenes and other organisms grown in specific medium relative to the total infected milk samples.*

Total infected milk samples	*Listeria monocytogenes* positive samples		Other organisms positive samples (*staphylococcus aureus* and *enterococcus*)	
No.	No.	%	No.	%
112	72	64.3	40	35.7

Table (3): *Differentiation between L. monocytogenes, staph. aureus and enterococcus by oxidase, catalase test and microscopical examination:*

organism	oxidase	catalase	Microscopical examination
Listeria monocytogenes	(-)	(+)	Gram positive bacilli, rod shape, arranged in short chains, sometimes in form of the letters V and Y
Staphylococcus aureus	(-)	(+)	Gram positive small round cocci forming a cluster or spherical shape, as grape-like clusters
Enterococcus Sp.	(-)	(-)	Gram positive ovoid cocci

(+) : positive result. (-) : negative result.

Table (4): *An incidence of L. monocytogenes relative to total number of examined milk samples.*

Total examined milk samples	Number of *Listeria monocytogenes* positive samples		Other organisms positive samples (*staphylococcus aureus* and *enterococcus*)	
No.	No.	%	No.	%
560	72	12.9	40	7.1

Table (5): *Biochemical identification of L. monocytogenes suspected isolates:*

Test	Result	Observation
Catalase test	(+)	Production of gas bubbles immediately (foaming).
Oxidase test	(-)	No change in color.
CAMP test	(+)	Arrow-shaped zone of weak enhanced hemolysis.

(+): positive result. (-) : negative result.

Depending on the present findings and its data analysis we can concluded that:

1 - It is important stand out that the number of positive samples in this study can be an important number due to that milk is consumed by inhabitants. The results of this study suggest that, there is a potential hazard for the population of the country.

2 - The presence of Listeria species in a variety of raw milk and dairy products indicate the potential risk of infection with Listerioses in people consuming raw milk, unpasteurized milk or traditional milk products. The presence of this pathogenic micro-organisms in raw milk and milk products is a particular concern because it has a health risk especially for pregnant women, elderly and immune-suppressed individuals. These high risk groups should avoid previously prepared unpasteurized dairy milk and milk products.

3 - This study has highlighted the urgent need for improved detection methods and techniques such as PCR and also improved new safety programs and epidemiological strategies to control *L. monocytogenes* and prevent its transmission to animal and human through milk and milk products.

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التوصيف الميكروبى لميكروب ليستريا مونوسيتوجين المعزول من الألبان

تم اجراء هذه الدراسة لعزل وتصنيف ميكروب ليستيريا مونوسيتوجين وذلك فى عينات مختلفة من اللبن والتى تم جمعها من مزراع ألبان مختلفة فى مصر ، حيث تم فحص عدد (560) عينة لبن ، وكانت الأغلبية العظمى منهم ما يعادل الـ (448) بنسبة (٨٠%) عينة فى صورة صحية سليمة.
وتم عزل ميكروب ليستيريا مونوسيتوجين من (72) عينة اللبن بنسبة (12.9%)، حيث خضعت جميع العينات للتصنيف البيوكيميائى الخاص بميكروب ليستيريا مونوسيتوجين.
وقد تم عمل تفاعل البلمرة المتسلسل لميكروب ليستيريا مونوسيتوجين عن طريق تعيين نوعين من الجينات وهم احد الجينات المعرفة لميكروب ليستيريا مونوسيتوجين Listeriolysin O gene (*hly*A) و p60 (*iap*) gene .