PCR TECHNIQUE FOR DETECTION OF SOME VIRULENCE ASSOCIATED GENES IN *LISTERIA MONOCYTOGENES* ISOLATED FROM TABLE EGGS AND CLINICAL HUMAN SAMPLES

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ABSTRACT

Received at: 31/12/2014

Accepted: 15/2/2015

The aim of this study was to assess the extent of *Listeria monocytogenes* in table eggs and its causation of human spontaneous abortions in Sohag city, Egypt by isolation methods and PCR analysis for the presence of virulence-associated genes. The result revealed that out of 30 pooled egg samples, 4 (13.3%) *L. monocytogenes* were isolated from egg shells only, among which 100% and 50% were revealed to encode *inlA* and *hlyA* virulence genes respectively, while recovered 5 (4.2%) *L. monocytogenes* strains from clinical human samples, among which each of *inlA* and *hlyA* virulence genes were present in 2 (40%) of the isolated strains. The results give a spotlight on the association between *L. monocytogenes* from food and some virulence genes with human abortion in Sohag city.

Key words: *Virulence genes, Listeria monocytogenes, Table eggs.*

INTRODUCTION

Listeria monocytogenes is a food borne pathogen causing listeriosis for human, due to its evidence in raw and ready to-eat (RTE) food products which are stored at refrigeration temperatures (Filiousis *et al.,* 2009; Pesavento *et al.,* 2010 and Shi *et al.,* 2015). Moreover, there have been several sporadic and epidemic outbreaks worldwide implicating *L. monocytogenes* contaminated foods (Ryser and Marth, 2007). *L. monocytogenes* is ubiquitous in nature; it can survive under conditions of high salt and low pH. Because it can grow even at low temperatures, it can be found in many kinds of foods during storage (Ivanek *et al.,* 2006). Ingestion of *L. monocytogenes* contaminated foods is associated with central nervous system (CNS) diseases, sepsis, endocarditis, focal infections, gastroenteritis, still births and abortions (Zhou and Jiao, 2004). The more severe form of listeriosis is invasive listeriosis with infections commonly occurring in vulnerable individuals like newborns, the elderly, immune suppressed patients and pregnant women (Rocourt *et al.,* 2000 and Hof, 2003). However, the degree of severity is dependent on the immunity of the infected individuals and the strain itself. Although listeriosis infections are rare, the fatality rate is as high as 20% - 30% annually (Allerberger, 2003; Yucel *et al.,* 2005 and Jeyaletchumi *et al.,* 2010). So, protective measures, such as the development of a surveillance and detection system concerning food safety are fundamental to ensure that raw and processed foods of animal origin as table eggs are safe for human consumption. Table eggs are one of the few foods that are used among the popular dishes consumed by the people at home, restaurants, and convenience stores in their natural states without artificial additives. Most freshly laid eggs are sterile, at least from inside in case of good flock management and absence of vertical transmission also by the presence of cuticle, shell membranes and the antimicrobial properties of eggs (Sayed *et al.,* 2009). But eggs may constitute a public health hazard, if contaminated with pathogens.

The traditional method for detecting this food-borne pathogen is time consuming; consisting of enrichment steps, plating on selective media and incubation for a period of time. Nowadays, molecular method by PCR has been so far the most extensively employed in various studies because it is a rapid procedure with both sensitivity and specificity for quick detection and identification of specific pathogenic bacteria from different sources (Sayed *et al.,* 2009; Dharmendra *et al.,* 2013 and Jamali *et al.,* 2013). Multiple key virulence factors such as internalin

(*inlA*), listeriolysin (*hlyA*), phosphatidylinositol phospholipase C (*plcA*), actin polymerization protein (*actA*) and invasive associated protein (iap) are important in *L. monocytogenes* pathogenesis (Furrer *et al.*, 1991 and Portnoy *et al.*, 1992). Therefore, detection of just one virulence associated gene by PCR is not always sufficient to identify *L. monocytogenes* (Nishibori *et al.*, 1995). In addition, it is plausible that some *L. monocytogenes* strain may lack one or more virulence determinants because of spontaneous mutations (Cooray *et al.*, 1994).

There is no official data on food poisoning/infection caused by *L. monocytogenes* in Egypt because *L. monocytogenes* is rarely tested in the food poisoning/infection cases. Nonetheless, the recent outbreaks of food borne listeriosis in USA and other countries and the high prevalence of *L. monocytogenes* in local foods (Jamali *et al.*, 2013) must draw the attention of local authorities on the possible widespread of *L. monocytogenes* in the country. In the present study, our goal was to put our hands on the incidence and the relation of *L. monocytogenes* in table eggs from local markets in Sohag city with spontaneous abortions in women, and to decipher the role of internalin (*inlA*) and listeriolysin (*hlyA*) in human listeriosis and to determine whether it could be used as readily assayable biomarkers to assess the pathogenic potential of strains that contaminate the food supply.

MATERIALS and METHODS

Samples:

1. Egg samples: Ninety fresh table eggs were collected during 2014 randomly from different markets in Sohag city, Egypt. Every three eggs from each market were represented as one egg pooled sample. All 30 pooled samples were examined for the incidence of *L. monocytogenes* in their shells and contents. Egg shells were tested by surface rinse method as described by Moats (1980) then egg contents were prepared and evacuated according to Speck (1976).

2. Human samples: a sum of 120 human samples including amniotic fluid (30), vaginal swabs (30), stool (30) and urine (30) were collected from 30 patients with a history of spontaneous abortions during second and third trimester of pregnancy. The samples were collected during 2014 from private and governmental hospitals in Sohag city, Egypt.

Microbiological analysis for *Listeria* isolation:

The ISO 11290 method was used for isolation and identification of *L. monocytogenes* in this study as described by Becker *et al.* (2006), as the first enrichment was done by using half Fraser broth which followed by second enrichment on Fraser broth followed by isolation on Oxford agar plates (Curtis *et al.*, 1989). Identification was performed according to FDA bacteriological analytical manual (Hitchins, 1995).

Genomic DNA extraction:

Listeria monocytogenes strains were grown overnight at 30°C onto HIBYE (Heart Infusion broth containing 0.5% of Yeast Extract), for sensitivity of PCR detection an overnight culture of *L. monocytogenes* was centrifuged at 12000 rpm for 10 min, washed once in phosphate buffered saline (PBS). From this suspension, 5 µl aliquot was directly used as a template for PCR amplification (Almeida and Almeida, 2000).

Detection of virulence genes:

The presence of internalin gene (*inlA*) was determined by PCR technique described by Almeida and Almeida (2000). PCR was performed in a reaction volume of 25 µl containing 10x PCR buffer [100 µM Tris (pH 9.0), 500 µM KCl, 15 µM $MgCl_2$, 0.1% gelatin] (Fermentas), 2 µM $MgCl_2$, 0.2 µM dNTPs, 40 pmol of *inlA*, 1 U of Taq DNA polymerase (Fermentas) and 3 µl of DNA template (50 µg/ml) under the following cyclic conditions: initial denaturation at 94 °C for 2 min, 30 cycles in sequence 94 °C for 20 s, 55 °C for 20 s and 72 °C for 50 s, and final extension at 72 °C for 2 min. The PCR technique described by Notermans *et al.* (1991) and subsequently modified by Kaur *et al.* (2007) was also used to detect *hlyA* gene. PCR was performed in 25 µl reaction mixture consisting of 10x PCR buffer (Fermentas), 2 µM $MgCl_2$, 0.2 µM dNTPs, 0.1 µM of forward and reverse primers of *hlyA* gene, 1.5 U Taq DNA polymerase (Fermentas) and 3 µl of DNA template (50 ng/µl) under the following cyclic conditions: initial denaturation at 95 °C for 2 min, 35 cycles in sequence 95 °C for 15 s, 60 °C for 30 s and 72°C for 90 s, and final extension at 72 °C for 10 min. The PCR products were analyzed by 1.5% agarose gel electrophoresis, stained with ethidium bromide, visualized by UV transilluminator and photographed. The details of oligonucleotide sequences (Pharmacia Biotech), used in this study are shown in the following table:

Sequences of primers used for detection of *inlA* and *hylA* virulence genes by PCR

Target gene	Primer sequence (5`→3`)	Amplicon size (bp)	Reference
inlA	5`- AGCCACTTAAGGCAAT- 3` 5`- AGTTGATGTTGTGTTAGA- 3`	760	Almeida and Almeida (2000)
hylA	5`- GCAGTTGCAAGCGCTTGGAGTGAA- 3` 5`- GCAACGTATCCTCCAGAGTGATCG- 3`	456	Kaur *et al.*(2007)

RESULTS

Table 1: Incidence of *L. monocytogenes* in table eggs samples using biochemical methods.

Positive samples	Table eggs					
	Egg contents		Egg shells		**Total**	
	No./30	%	No./30	%	No./30	%
L. monocytogenes	0	0	4	13.3	4	13.3

Table 2: Incidence of *L. monocytogenes* in human samples using biochemical methods.

Positive samples	Human Samples									
	Amniotic fluids		Vaginal swabs		Urine		Stool		**Total**	
L. monocytogenes	No./30	%	No./30	%	No./30	%	No./30	%	No./120	%
	2	6.6	2	6.6	1	3.3	-	-	5	4.2

Table 3: Pathogenicity and PCR profiles of *L. monocytogenes* isolates from table eggs and human.

Virulence genes	Egg shells isolates					Human isolates					
	1	2	3	4	**Total %**	Amniotic fluid		Vaginal swab		Urine	**Total %**
						P1	P2	P1	P2	P2	
inlA	+ve	+ve	+ve	+ve	100%	+ve	-ve	+ve	-ve	-ve	40%
hylA	-ve	+ve	+ve	-ve	50%	+ve	-ve	+ve	-ve	-ve	40%

P1= First patient
P2= Second patient

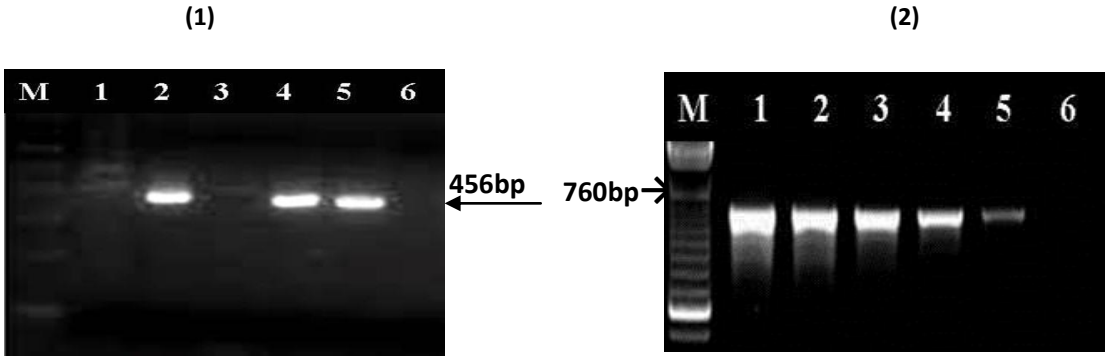

Figure (1): Agarose gel electrophoresis of PCR amplification products using specific listeriolysin gene (*hlyA* primers) of *L.monocytogene*s isolated from eggs. **Lane M:** 100 bp ladder as molecular DNA marker, **Lane 1**: Control negative, **Lane 2**: Control positive, **Lane 3** and **Lane 6**: Negative *L.monocytogenes* for listeriolysin production, Lane **4** and **Lane 5**: Positive *L.monocytogenes* for listeriolysin production.

Figure (2): Agarose gel electrophoresis of PCR amplification products using specific primers of *InlA* gene of *L.monocytogene*s isolated from eggs. **Lane M:** 100 bp ladder as molecular DNA marker, **Lane 1**: Control positive for *InlA* gene, **Lane 6**: Control negative, **Lane 2**, **Lane 3, Lane 4** and **Lane 5**: Positive *L.monocytogenes* for *InlA* gene.

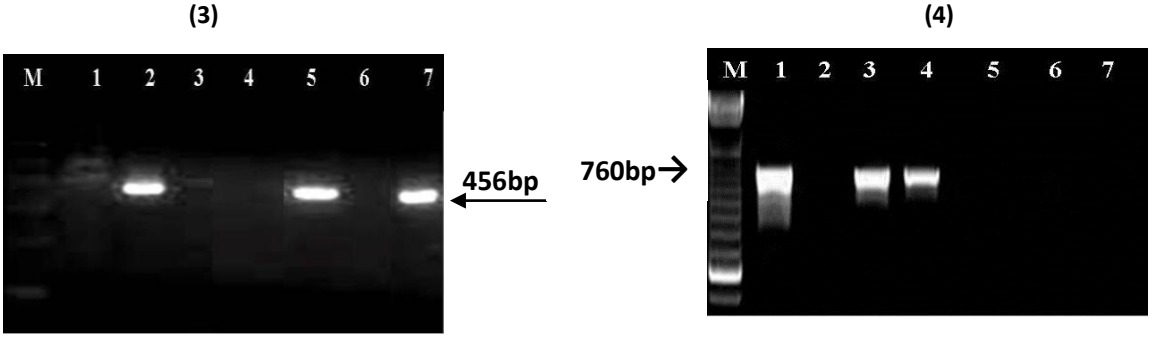

Figure (3): Agarose gel electrophoresis of PCR amplification products using specific listeriolysin gene (*hlyA* primers) of *L.monocytogene*s isolated from clinical human samples**. Lane M:** 100 bp ladder as molecular DNA marker**, Lane 1:** Control negative**, Lane 2:** Control positive**, Lane 3, Lane 4** and **lane 6:** Negative *L.monocytogenes* for listeriolysin production, **Lane 5** and **Lane 7: Positive** *L.monocytogenes* for listeriolysin production**.**

Figure 4: Agarose gel electrophoresis of PCR amplification products using specific primers of *InlA* gene of *L.monocytogenes* isolated from clinical human samples**. Lane M:** 100 bp ladder as molecular DNA marker**, Lane 1:** Control positive for *InlA* gene**, Lane 7:** Control negative**, Lane 3** and **Lane 4:** Positive *L.monocytogenes* for *InlA* gene**, Lane 2, Lane 5** and **lane 6:** Negative ***L.monocytogenes*** for *InlA* gene**.**

DISCUSSION

The microbiological and biochemical analysis of 30 pooled egg samples in the present study revealed failure of detection of *L. monocytogenes* from all egg contents samples (Table 1), this result goes parallel with that of Saad and El-Prince (1995); Korashy *et al.* (2008); Sayed *et al.* (2009); Ghasemian-Safaei *et al.* (2011) and Namish (2011). Absence of *L. monocytogenes* in egg contents may be attributed to the unsuitability of pH of raw egg albumen for growth of *L. monocytogenes*. Furthermore, presences of antibacterial properties of eggs which hydrolyze the polysaccaride bacterial cell wall causing cell lysis (Yadava and Vadehra, 1977). Table 1 showed that the occurrence of *L. monocytogenes* in the egg shells samples was 4(13.3%) which is lower than the result of Saad and El-Prince (1995) (17.7%) and Jones *et al.* (2004) (18.3%) and higher than Sayed *et al.* (2009) (7%). While, each of Korashy *et al.* (2008) and Ghasemian -Safaei *et al.* (2011) failed to isolate *L. monocytogenes* from the examined egg shells samples. Regarding the increasing consumption of egg and its products, it is necessary to investigate egg contamination with *L. monocytogenes* as it is very frequently present in broiler poultry farms and flocks of laying hens (Chemaly *et al.*, 2008), the egg shell

contamination may be resulted from deposition of fecal material on the shell, ovarian or oviduct and gut flora, egg crates, packing and storage, clothes and hands of poultry workers, dust, transporting and marketing (De Reu *et al.*, 2006).

About one-third of reported human listeriosis cases happen during pregnancy, which may result in spontaneous abortion in second or third trimester (CDC, 2005). In the present study, the microbiological and biochemical analysis of 120 samples from 30 patients with a history of spontaneous abortions revealed 5(4.2%) isolates resembling *L. monocytogenes*; two samples (6.6%) from amniotic fluid, two samples (6.6%) from vaginal swabs and one (3.3%) urine sample, while did not recover from stool samples (Table 2). In contrast, Dhanashree *et al.* (2003) could not isolate *L. monocytogenes* from amniotic fluids. However, Hanan (1994); Asmaa (1998); Kaur *et al.* (2007); Stepanovic *et al.* (2007); Namish (2011) and harmendra et al. (2013) reported lower results of positive infection of *L. monocytogenes* in vaginal swabs 2.08%, 3.3%, 1.6%, 0.1%, 2% and 1.3% respectively. In contrast higher results (10%) in vaginal swabs were recorded by Stephen *et al.* (1978). Kaur *et al.* (2007) showed that 1.6% of urine samples were infected with *L. monocytogenes*, while 0% from stool samples as well as Dhanashree *et al.* (2003).

Lamont *et al.* (2011) illustrated that *L. monocytogenes* is an intracellular pathogen which, following ingestion of contaminated food, is phagocytosed and internalised in epithelial cells by the interaction between the bacterial surface protein, internalin (*inlA*), and its receptor on the epithelial surface (E-cadherin.). The organism is vacuolated by macrophages, polymorphonuclear leucocytes and other plasma cells, and escapes the vacuole through the action of listeriolysin (*hlyA*), finally entering the cytoplasm where proliferation occurs. So; several polymerase chain reaction systems (PCR) for the detection of *L. monocytogenes* have been described, mainly targeting the virulence genes such as those encoding the *inlA* and *hlyA* (Dharmendra *et al.*, 2013). However, it is plausible that some *L.monocytogenes* strain may lack one or more virulence determinants because of some mutation (Cooray *et al.*, 1994). Using PCR assay for detection of single virulence associated gene is neither sufficient to identify the *L. monocytogenes* isolates nor to reveal its true pathogenic potential as majority of *L. monocytogenes* isolates showed different gene profiles (Rawool *et al.*, 2007).

Results illustrated in Table 3, figure 1 and figure 2 revealed that all four (100%) isolates of *L. monocytogenes* isolated from the examined egg shell samples were found to harbor *inlA* gene, while 2 (50%) of them posses *hlyA* gene. In contrast, Namish (2011) failed to detect *inlA* gene in *L. monocytogenes* isolated from examined egg shell samples. Jacquet *et al.* (2004) and Roche *et al.* (2009) stated that some isolates of *L. monocytogenes* isolated from different raw and ready to eat food were harbor *inlA* gene with or without *hlyA* gene which supported the usefulness of studying the pathogenic potential of strains that contaminate the food supply.

Table3, figure 3 and figure 4 shows that two out of five *L. monocytogenes* strains isolated from the clinical human samples posses virulence genes (from amniotic fluid and vaginal swabs) which recovered from one patient. Each of two strains were associated with both *inlA* and *hlyA* virulence genes, which confirm the finding that internalin and listeriolysin are highly associated with clinical strains which support their critical role in human listeriosis (Lamont *et al.*, 2011). Burtun and Blais (2006) and Dharmendra (2013) found that *inlA* and *hlyA* virulence genes were represented in each of clinical human samples and food samples. Moreover, the presence or absences of some virulence genes are good indicators of the level of virulence of *Listeria* strains (liu *et al.*, 2007). The current study also reveals that internalin and listeriolysin can be used as biomarkers for stratifying the virulence of strains recovered from food surveillance program.

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الكشف عن بعض جينات الضراوة فى ميكروبات الليستيريا مونوسيتوجنز المعزولة من بيض المائدة والانسان باستخدام اختبار البلمرة المتسلسل

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تم تجميع ٩٠ عينة من بيض المائدة من محلات البقالة بسوهاج و١٢٠ عينة من سيدات تعانى من اجهاض متكرر بواقع ٣٠ عينة من المهبل ،السائل الأمنيوسى ،البراز والبول بمدينة سوهاج لمعرفة مدى تواجد ميكروب الليستريا مونوسيتوجنز، ومعرفة مدى تواجد بعض جينات الضراوة فى العترات المعزولة من جميع العينات. وقد تبين من الفحص البكتريولوجى أن 13.3% من عينات قشر بيض المائدة و٤.٢% من عينات المرضى المختلفة كانت ملوثة بميكروب الليستريا مونوسيتوجنز. كما تم فحص العترات المعزولة لبعض جينات الضراوة باستخدام اختبار البلمرة المتسلسل وأوضحت النتائج وجود بعض جينات الضراوة تحت الإختبار فى العترات المعزولة من البيض والمرضى بنسب مختلفة. ومن هذه الدراسة نوصى الجهات المختصة بضرورة وضع بروتوكول لإيجاد علاقة بين مسببات حالات التسمم والإجهاض المفاجئ.