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MOLECULAR DETECTION OF *LISTERIA MONOCYTOGENES* PATHOGENIC GENES AS FOOD POISONING MICRO-ORGANISM ISOLATED FROM READY- TO- EAT MEALS IN PORT-SAID CITY MARKETS

(With 3 Tables and 5 Figures)

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الكشف الجزيئي للجينات مسببة المرض لميكروب الليستيريا مونوسيتوجينز كأحد ميكروبات التسمم الغذائي المعزولة من الوجبات الجاهزة من أسواق مدينة بورسعيد

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تم تجميع ١٠٥ عينة من الوجبات السريعة (١٥ عينة لكل من الكفتة، الكبدة، اللانشون، السجق، هوت دوج، البسطرمة وجبنه مطبوخة بالبسطرمة) من المطاعم المختلفة وبائعي الوجبات السريعة بمدينة بورسعيد. وأجري الفحص البكتيريولوجى على العينات حيث أفضت إلي وجود ميكروب الليستيريا مونوسيتوجينز بنسبة ٢٦,٦% من عينات البسطرمة فقط بينما كانت جميع العينات الأخرى سلبية لميكروب الليستيريا مونوسيتوجينز. وقد وجد جين الضراوة (بى ار اف ايه) فى جميع المعزولات وهو المسؤل عن بداية إحداث المرض. هذا وقد تمت مناقشة الأهمية الصحية لهذا الميكروب ومدى تأثيره على الصحة العامة وصحة الإنسان وكذلك الشروط الواجب توافرها وإتباعها للمحافظة على سلامة الوجبات السريعة.

SUMMERY

Listeria monocytogenes is a major safety concern for ready- to- eat (RTE) meals, which are frequently contaminated with this pathogen. So a total of 105 random samples represented by 7 kinds of ready –to-eat meals (kofta, fried liver (kibda), luncheon, sausage, hotdog, cooked cheese with basturma and basturma) were collected from different

supermarkets and street venders in Port-Said City. *L. monocytogenes* was isolated from basturma only in 4 samples (26.6%). On other hand *L. monocytogenes* could not be isolated from any of the other examined samples. Polymerase chain reaction is a powerful technique for detection of pathogens in foods. It is a rapid procedure with both sensitivity and specificity for quick detection and identification of specific pathogenic bacteria from different sources. *Listeria monocytogenes* detection methods based on PCR amplification of the *prfA* and *hly* genes sequences have been reported. The virulence gene *prfA* was found in all isolates which is responsible for the initiation of pathogenesis so its target is to detect pathogenic *L. monocytogenes*. The public health hazard of this microorganism as well as recommended measures to improve quality status of ready- to- eat (RTE) meals was discussed.

Key words: *Listeria monocytogenes detection, ready- to- eat meals, Food-borne disease, PCR, Public health.*

INTRODUCTION

In recent years, there was an increased trend for RTE meals to be consumed both at home and restaurants. RTE meals such as kofta, fried liver (kibda), luncheon, sausage, hot dog, cooked cheese with basturma and basturma have become more popular RTE meals in Middle East countries. They considered the most common RTE sold by street vendors and fast food restaurants (Cressey and Lake, 2005; Vorst *et al.*, 2006; Acciari *et al.*, 2011).

Because of the changing life style in Egypt, people consume a variety of fast foods on a daily basis. Traditional meals- containing fast foods such as kofta, fried liver (kibda) sandwiches became more popular (Hamedy *et al.*, 2007).

Food-borne infections are important public health concern worldwide. According to reports of the World Health Organization and the Center for Disease and Prevention, every year a large number of peoples are affected by food borne diseases due to consumption of contaminated beef (Busani *et al.*, 2005; Cressey and Lake, 2007).

Meat constitutes the most important items of human food because of its palatability and high nutritional value, at the same times, meat is prone to contamination from the hands and clothes of staff, knives and other equipments occurred post production or

recontamination during further handling (Gill *et al.*, 2001; El-prince and Sayed, 2004).

Listeriosis is a foodborne bacterial disease caused by *L. monocytogenes*, that is widely spread throughout the environment. It had been isolated from soil, water, silage, and many other environmental sources. The organism can grow in PH range of 4.4°C – 9.4°C. resists high salt levels, nitrite, acid and relatively resistant to drying, but easily destroyed by heating and it can grow between 0°C- 45°C. (Janny, 2007; Sue *et al.*, 2009). *L. monocytogenes* was found in at least 37 species of mammals, both domestic and wild, as well as up to 10% of human may be intestinal carriers. It had been detected in 17 species of birds, some species of fish and shellfish, and is especially pathogenic to high risk populations, such as newborn, pregnant women, elderly, and immunocompromised individuals (Mugampoza *et al.*, 2011).

Although listeriosis is relatively uncommon, it is a potentially fatal disease. It frequently result in abortions in pregnant women. Even though the symptoms may be relatively mild in the mother, the illness may be transferred to the fetus causing serious illness or fetal death. Some symptoms of *L. monocytogenes* may include meningitis, encephalitis, septicemia, spontaneous abortion, still birth, and influenza-like symptoms (William and Catherine, 2001; Sutherland *et al.*. 2003: Azevedo *et al.*, 2005; Sue *et al.*, 2009).

Detection of *L. monocytogenes* by molecular methods is very specific and can be as fast as the immunological assays (Janzten *et al.*, 2006). A number of PCR assays had been described for its detection in foods (Levin, 2003). PCR methods had superior sensitivity when compared to standard nucleic acid probes or immunoassays. However, complex sample preparation methods and the use of gel electrophoresis endpoint detection have hampered the transition of these methods from research to routine use in food microbiology laboratories. Nevertheless, factors influencing the performance of conventional PCR in foods continue to be investigated (Aznar and Alarcón, 2003) and standardized (D'Agostino *et al.*, 2004).

Sequence comparison of individual genes in the *prfA* virulence gene cluster (*pVGC*), a central virulence gene cluster, from different *Listeria* species indicated that priming sites within the genes appeared to be specific for *Listeria monocytogenes* exclusively. Therefore, the pVGC was targeted for polymerase chain reaction (PCR) assays to detect and specifically identify *L. monocytogenes*. Each gene of the

pVGC was specifically amplified in *L. monocytogenes* but not in other *Listeria* species (Jung *et al.*, 2009).

This study was planned to investigate the presence of *L. monocytogenes* among some selected ready- to- eat (RTE) meals using the classical methods of isolation in comparison with molecular tools. It also aimed to detect one of the virulence genes cluster (*pVGC*) responsible for initiation of pathogenesis (*prfA* gene).

MATERIALS and METHODS

A - Collection of Samples:

A total of 105 random samples of RTE meals included fried liver (kibda), kofta, sausage, hotdog, luncheon, cooked cheese with basturma and basturma from different supermarkets and street vender in Port-Said City for detection of *L. monocytogenes*.

B - Isolation:

Twenty-five grams of each RTE meals samples were homogenized in a stomacher for 2 min in 225 ml of *Listeria* enrichment broth (Difco), and incubated at 30°C for 48 hr. the samples were divided into 2 portions, one for molecular identification and the other to continue the isolation. After incubation one loopful was subcultured on *Listeria* Oxford medium base. The plates were incubated at 35°C for 24-48 h.

C - Identification:

Five typical colonies were transferred from *Listeria* Oxford medium base to Trypticase soy agar with yeast extract for purification. Purified isolates were identified by the Gram- stain, Catalase test, motility test, biochemical tests and Christie- Atkins, Munch- Petersen; test of haemolysis (CAMP Test). Further confirmation of *L. monocytogenes* the isolates were inoculated in to 10% aqueous stock solution of Manitol, L. Rhamnose and D. Xylose (FDA, 2003).

D - Polymerase chain reaction (PCR)

DNA Extraction: Boiling method **(Bansal, 1996).** Bacterial pellets were washed once with 1 ml phosphate buffered saline (PBS), pH 7.4, resuspended in a same volume of cold water and incubated in a boiling water bath for 10 min. The clear supernatants obtained after a 5 min centrifugation at 12000g were used for PCR reaction.

Oligonucleotide Primers: in this study 2 sets of primers were used, *hyl* gene specific for conformation of *L. monocytogenes* and not any other

type of *Listeria* and *prfA* gene the initiator gene for pathogenesis. The sequence, cycling conditions and amplicone size were described in table (1). The PCR products were visualized on 1.3% agarose gel in 1x TBE using GeneRuler 100 bp plus DNA Ladder (Fermentas Cat. No. #SM0323).

Table 1: Shows the sequence, cycling conditions and amplicone size of the used genes:

Gene	Sequence 5\'- 3\'	Cycling condition	Product size	Reference
hyl	LM1 CCT-AAG -ACG-CCA-AT C-GAA LM2 CCT-AAG -ACG-CCA-AT C-GAA	Initial denaturation 95°C for 5 min 30 cycle of 95 °C for 15 sec 57 °C for 2 sec 72 °C for 30 sec Final extention at 72 °C for 5 min	702 bp	Mengaud et al (1988)
prfA	prfA-A CTG-TTG-GAG - CTC-TTC-TTG- GTG-AAG -CAA-TCG prfA-B AG C-AAC-CTC-GGT-ACC- ATA -TA C- TAA -CTC	Initial denaturation 95°C for 5 min 30 cycle of 95 °C for 15 sec 60 °C for 30 sec 72 °C for 90 sec Final extention at 72 °C for 5 min	1060 bp	Wernars et al (1992)

RESULTS

In the present study, all types of RTE food examined (105 samples listed in Table 2 and Fig. 1) were negative to the presence of *Listeria* except that of Basturma, four samples out of the examined fifteen were positive by isolation with an incidence rate of 26.6% and overall recovery rate of 3.8%. All *Listeria* spp. hydrolyze esculin and the inclusion of esculin and ferric iron in enrichment or plating media results in the formation of an intense black color (Fraser and Sperber, 1988). This is due to the complexation of the ferric iron with 6, 7-dihidroxycoumarin, the product of esculin cleavage by β-D-glycosidase, resulting in a black precipitate.

Table 2: Incidence of *L. monocytogenes* in the examined samples of RTE meals.

Types of samples	No. of samples examined	Positive samples of *L. monocytogenes*	
		No	%
fried liver(kibda)	15	0	0
Kofta	15	0	0
Sausage	15	0	0
Hotdog	15	0	0
Luncheon	15	0	0
cooked cheese with basturma	15	0	0
basturma	15	4	26.6
Total	105	4	3.8

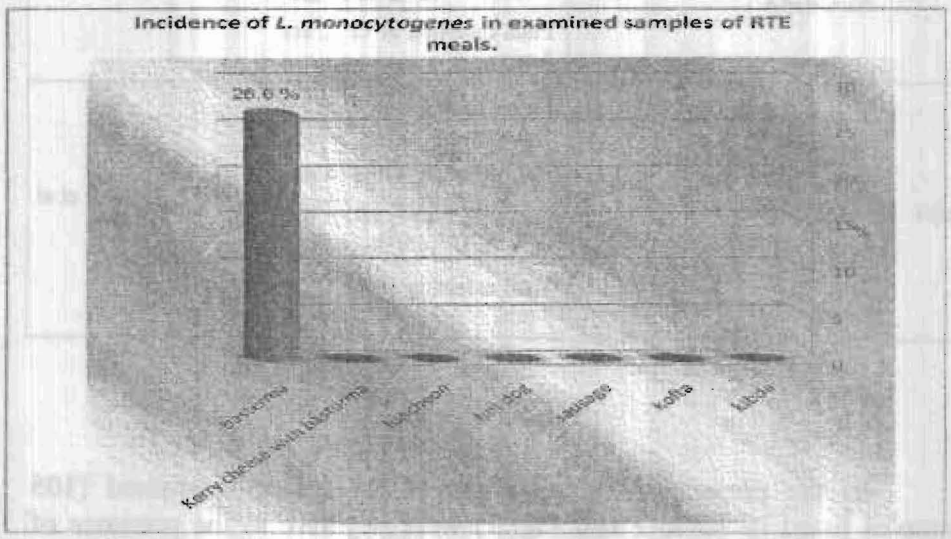


Fig. 1: Incidence of *L. monocytogenes* in the examined samples of RTE meals.

These isolates were subjected to hemolysis, Christie- Atkins, Munch- Petersen; test of haemolysis (CAMP Test) and acid production tests for differentiation of *listeria* species as listed in Table 3 and Fig 2. An "arrowhead" hemolysis is seen between growth of *Listeria monocytogenes* and Group B *Staph aureus*. This is because of alpha toxin produced by *Staph aureus* interacts with Christie- Atkins, Munch-Petersen; test of haemolysis (CAMP Test) factor and produce synergistic hemolysis.

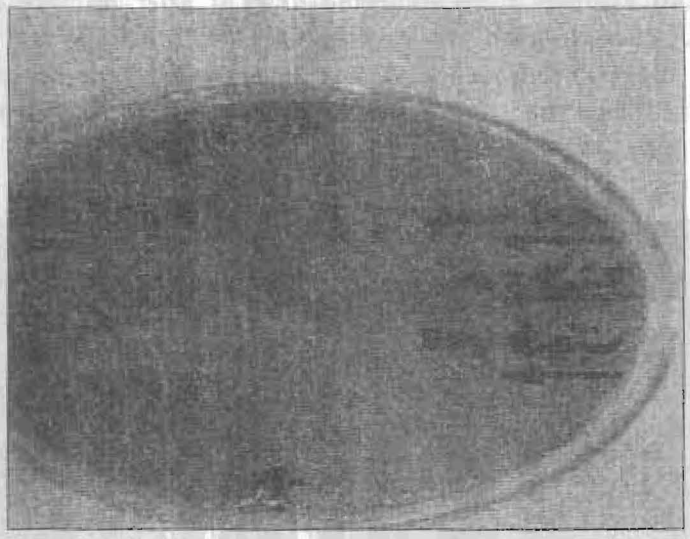


Fig. 2: *Listeria monocytogenes* on blood agar Christie- Atkins, Munch-Petersen; test of haemolysis (CAMP Test) showing the characteristic arrowhead hemolysis between basturma isolates only and *Staph aureus*.

Table 3: Biochemical differentiation of *Listeria* species in the examined isolates:

Listeria spp.	Haemolysis		CAMP		Acid production from					
					D-Xylose		L-Rhamnose		Mannitol	
	C	T	C	T	C	T	C	T	C	
L. monocytogenes	+	+4	+	+4	+	+4	-	-	V	
L. seeligeri	+	-	+/-	-	-	-	-	-	-	
L. ivanovii	++	-	-	-	+	-	-	-	-	
L. innocua	-	-	-	-	-	-	V	-	V	
L. welshimeri	-	-	-	-	-	-	-	-	+	
L. grayi	-	-	-	-	+	-	+	-	V	

+:positive, +/–: weakly positive, –: negative, CAMP: Christie- Atkins, Munch- Petersen; test of haemolysis, V: variable
C= control, T = tested isolates

All samples were subjected to PCR after primary enrichment, The PCR results didn't give any results after first PCR run from initial culture (Fig. 3) compared to *L. monocytogenes* reference strain; by re-PCR only basturma gave a characteristic band at 702 bp to *hyl* gene specific for *L. monocytogenes* (Fig. 4).

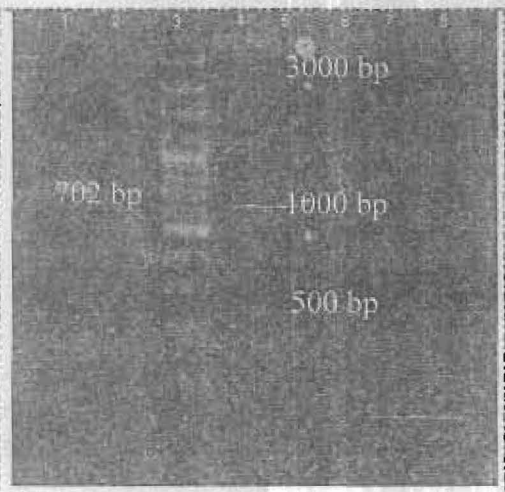


Fig. 3: Showed the electrophoretic pattern of PCR products of primary culture of *L. monocytogenes* from tested RTE food compared to Reference strain using *hyl* gene

1- *L. monocytogenes* Reference strain
2- Basturma Isolate
3- Marker (Fermentas)
4- Luncheon
5- Hotdog
6- Sausage
7- Kofta
8- Fried liver(kibda)

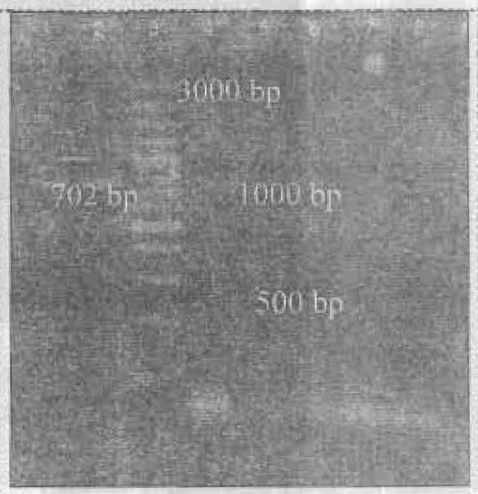


Fig. 4: Showed the electrophoretic pattern of PCR products of Re-PCR products from tested RTE food compared to Reference strain using *hyl* gene

1- *L. monocytogenes* Reference strain
2- Basturma Isolate
3- Marker (Fermentas)
4- Luncheon
5- Cooked cheese with basturma
6- Sausage
7- Kofta
8- Fried liver(kibda)

The positive PCR samples for *hyl* gene were examined for the presence of *prfA* gene and gave the characteristic band at 1060 bp compared to reference strain (Fig 5) also at the 2nd round PCR (Re-PCR).

Fig. 5: Showed the electrophoretic pattern of PCR products from tested RTE food compared to Reference strain using *prfA* gene

1 - Luncheon

2 - Cooked cheese with basturma

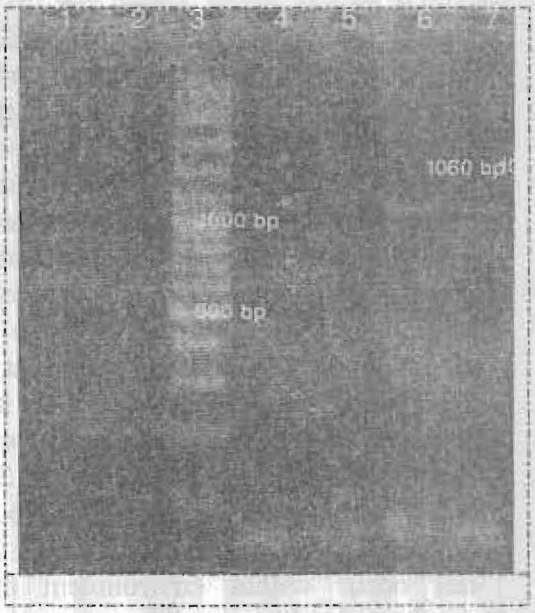
3 - Marker (Fermentas)

4 - Sausage

5 - Kofta

6 - Basturma Isolate

7- *L. monocytogenes* Reference strain



DISCUSSION

The microbiological safety of food products is of extreme importance to industry, public health officials, regulatory agencies and the public little information is available regarding the occurrence of *L. monocytogenes* in meat, meat product and soft cheese in retail market in Port-Said City. This survey provided a clear picture for the prevalence of *L. monocytogenes* in RTE meals offered to consumers. *L. monocytogenes* was isolated from 4 basturma samples with a percentage of (26.6%) in Table 2 and figure 3. In this concern, isolation of *L. monocytogenes* in final products of basturma were have been reported by Steven *et al.* (2006).

An additional microbiological hazard of potential concern in basturma is *Listeria monocytogenes*. Basturma is sold as a raw product and some people may consume basturma as it is, while cooking by the consumer is important for preventing food borne illness due to listeriosis associated with this product. *L. monocytogenes* growth on the finishing product is unlikely because of its reduced water activity (Aw) Guidance from USDA states that, decreasing a products water activity to < 0.92 and PH <4.39 will prevent *L. monocytogenes* growth (USDA, 2004). The American Meat Institute Foundation (2005) stated that unless the finishing basturma (contained *L. monocytogenes* at levels greater than 4 log CFU, or supported growth to such a level), cooking procedures

should dramatically decrease the risk of basturma -linked Listeriosis. In general, the organism has been found in raw milk, soft cheese, fresh and frozen meat and vegetable products (Hege *et al.*, 2000)

The ability of food borne micro-organisms as *L. monocytogenes* to become disseminated from naturally contaminated foods to various hand and food contact surface is well know. The results achieved from Tables 2and 3 revealed that, *L. monocytogenes* could not be detected in fried liver (kibda), kofta, Luncheon, cooked cheese with basturma, sausage or hot dog samples. The incidence of *L.monocytogenes* in luncheon meat was Lower than that obtained by Gombas *et al.* (2003) who found it in 0.89% of luncheon meat. In contrast, *L. monocytogenes* could not be isolated from meat samples (Elgazzar and Sallam, 1997; Mohamed; Ali, 1999; Saad *et al.*, 2001). This wide variation may be explained in terms of geographic location, isolation methods and kinds of media employed (Akpolat *et al.*, 2004). Cross-contamination, which can occur within environment of food-processing equipment, is considered to be a possible source of *Listeria* contamination in processed meat such as luncheon. *L. monocytogenes* is able to attach to and survive on various working contact surfaces (Borucki *et al.*, 2003). One reason may be its ability to form biofilms (Wong, 1998). Furthermore, during further transformation processes of raw meat into meat products *L.*monocytogenes can be introduced, where the amount depends on the extent of cross-contamination, personal and general hygienic measures and the process parameters (Glass and Doyle, 1989). In addition, minced/chopped meat products as luncheon, by their nature, undergo extensive processing and handling during their production. This leads to greater opportunities for *L. monocytogenes* contamination (Tompkin *et al.*, 1992 and Uyttendaele, 1997). The difference occurring, either increase or decrease in the incidence of other literatures than that obtained results, this attributed to the nature processing load to RTE meals, also due to the level of contamination of other additives, which added to the RTE meals and difference in country and type of sample analysis.

The mortality rate of listeriosis is very high, approximately 30% (Griffiths, 1989), and for this reason the Food and Drug Administration maintains a policy of zero-tolerance for *L. monocytogenes* (Anon, 2003). With this concern, the ability to detect this pathogen at low levels is considered essential because most of the foods susceptible to contamination are ready-to-eat products, which are not cooked or otherwise processed before consumption. These considerations underline

the benefit of monitoring sensitive *L. monocytogenes* to ensure the microbiological quality of foods and to reduce risks for public health.

The smoking, cooking, fermentation or drying processes used to make the samples tested can be considered effective antimicrobial processes for control of *L. monocytogenes* on RTE meals (Steven *et al.*, 2006). The European community directive on milk and milk-based products specifies zero tolerance for soft cheese and the absence of the organism in 1 g of the product (Food Safety and Inspection Service, 2000).

Current microbiological culture methods rely on growth in culture media, followed by isolation, and biochemical and serological identification. However, the detection of this pathogen in food by these standard culture methods is made difficult by the sporadic or low levels of contamination (<100 cfu/ g) can't be relied incase of the presence of a high level of background micro-flora and competitor organisms that could mask the presence of *L. monocytogenes*, or even interference due to food matrix components (Norton *et al.*, 2001). Moreover, these methods are laborious and time consuming, requiring a minimum of five days to recognize *Listeria* spp. and about 10 days to identify *L. monocytogenes* by confirmatory tests (Anon, 1996), while immediate action should be taken in case of contamination since it is of fundamental importance to ensure the safety of food products, especially the case of those food matrices having short shelf-lives, such as meat or dairy products.

In the past years, advancements in biotechnology have resulted in the development of rapid methods that reduce analysis time and offer great sensitivity and specificity in the detection of pathogens. Among these, PCR has been increasingly used for the rapid, sensitive and specific detection of foodborne pathogens (Olsen *et al.*, 1995). However, the successful application of PCR assays to food samples has been hindered by the lack of a rapid and efficient method for the preparation of PCR-amplifiable DNA (Lantz *et al.*, 1994). The presence of PCR inhibitors in food samples (Rossen *et al.*, 1992; Bickley *et al.*, 1996) represented the main limitation in this kind of assays due to the production of false negative results. For this reason, the application of PCR-based methods is closely linked to the selection of suitable methods for DNA extraction.

The results obtained from isolation were in line with that of molecular diagnosis as PCR detected only the presence of *L. monocytogenes* in 4 basturma samples at 2nd PCR. This clarify the

reason why PCR didn't give any result at first time, but gave results with Re-PCR during the detection of *hyl* and *prfA* genes.

Gouws and Liedemann (2005) and Amagliani *et al.* (2007) associated the main limitation with PCR application to food-contaminating microorganisms concerns to the presence of inhibitory substances that are coextracted with DNA and may be present in the sample, causing a failure in the amplification reaction which leads to false negative results. Therefore, quality and purity of extracted nucleic acids are the main requirements for a PCR-based detection assay at the mean while the selection of a proper extraction method is determinant for a successful and valid PCR analysis.

This consideration could explain the results obtained for samples extracted using the boiling method suggested by Bansal *et al.* (1996), showing faint signals in gel analysis. Although lysis by boiling appeared to be the most convenient extraction protocol, particular care should be taken when food contamination with very low bacterial count is suspected. Cooray *et al.* (1994) noted that boiling for 5 min at 100°C was superior to heating at 95°Cfor releasing DNA from cells.

Oligonucleotide primers targeted to the *L. monocytogenes hlyA* gene were highly species specific and provide a means for easily differentiating *L. monocytogenes* from other hemolytic species of *Listeria* (Dencer and Boychuk, 1991). Several genes coding for various aspects of virulence in *L. monocytogenes* had been identified and used for PCR detection of the organism. Wernars *et al.* (1992) made use of the *prfA* gene to develop a highly specific PCR for recognition of pathogenic *L. monocytogenes* strains. The primers used, *prfA-A/ prfA-B*, flank a 1060-bp sequence encompassing the entire prfA gene directed against nucleotides 181 to 207 and 1462–1482 of the *prfA* gene sequence, were found to be specific for all virulent strains of *L. monocytogenes* tested, and readily distinguished them from a-virulent strains of *L. monocytogenes* and representatives of the other six *Listeria* species (Levin, 2010).

In conclusion, this study has demonstrated the presence and distribution of *L. monocytogenes* in a variety of RTE meat products in Port-Said. PCR was more rapid and sensitive method, useful for the rapid detection of *L. monocytogenes* in a reasonable period of time which may avoid costly recalls and reducing outbreaks. As well as any ready-to-eat foods such as hot dogs, luncheon meats, cold cuts, fermented and dry sausage should be eaten only after they have been reheated to a high temperature.

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