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ORIGINAL ARTICLE

Detection of *Listeria* Species in Farm Milk and White Soft Cheese

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Key words:

Listeria monocytogenes, Raw milk, Listeriosis, Oxford medium, Enrichment, Polymerase chain reaction, Prevalence, Pasteurization

ABSTRACT

Background*: Listeria is one of the food-borne pathogens common contaminant in dairy products. Consumption of milk and dairy products, in particular soft cheese often implicated as the source of infection in severe outbreaks of listeriosis.* ***Objective****: This work aimed to investigate the prevalence of Listeria monocytogenes and other Listeria spp. in raw milk and white soft cheese by conventional methods and polymerase chain reaction.* ***Methodology****: Two hundreds samples (50 of each market raw milk, individual farm milk and Kariesh cheese plus 25 of each Damietta cheese and Talaga cheese) where randomly collected from dairy farms, different shops and supermarkets in Mansoura City, Dakahlia governorate. Direct isolation of Listeria on Oxford media was performed and compared with indirect (enrichment) method followed by biochemical identification. Polymerase chain reaction was done for accurate detection of Listeria monocytogenes.* ***Results****: The prevalence of Listeria monocytogenes by direct method was 10%, 8%, 8% and 4% in market raw milk, Kariesh cheese, Talaga cheese and Damietta cheese, respectively. On the other hand, indirect (enrichment) isolation of Listeria monocytogenes showed prevalence of 8% and 4% in market raw milk and Talaga cheese, respectively. Listeria ivanovii and Listeria Seeligeri were also detected as 24% and 12% in market raw milk, 24% and 30% in individual farm milk, 16% and 32% in Kariesh cheese, 48% and 36% in Talaga cheese and 24% and 36% in Damietta cheese by direct method. On the other hand, by indirect (enrichment) method the prevalence of Listeria ivanovii and Listeria seeligeri was 14% and 2% in market raw milk, 6% and 14% in individual farm milk, 12% and 12% in Talaga cheese and 12% and 16% in Damietta cheese, but Kariesh cheese was free form Listeria ivanovii and Listeria seeligeri was 4%.* ***Conclusions****: The general principles of food hygiene should still be enforced in order to minimize count of Listeria monocytogenes in milk and dairy products during the handling, storage and manufacturing in traditional dairies. Control of the feeding cattle and milk pasteurization can also limit the contamination with Listeria monocytogenes.*

INTRODUCTION

Milk has been used for human consumption for thousands of years. People drink milk of many animals including cows, camels, goats, reindeer, sheep and water buffalo. Milk is naturally nutrient-rich and balanced with a unique proportion of carbohydrates and protein in addition to the bone-boosting calcium, phosphorus and vitamin D[1].

Cheese from ancient times until today is an excellent solution for long time preservation of milk. Although there are several varieties of cheese available in different texture, taste and smell, all types retain some basic characteristics that make them valuable in human diet. Milk in addition to being considered of high nutritive value to mankind, it is an excellent medium for growth of microorganisms[1].

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Milk can be contaminated at any point in the milk production process. The sources of contamination are multiple and the pathways are complex. Contamination of milk can occur directly by dairy animals shedding pathogens into milk, or indirectly by contamination of milk during the milking process, collection and transportation. Other sources of environmental contamination are water, pests, soil, faces and pets. Infected farmers, not respecting hand hygiene, are also a potential source of contamination of milk[2].

Listeria is one of food-borne pathogens common contaminant in the dairy environment. Consumption of milk and dairy products, in particular soft cheeses, often implicated as the source of infection in severe outbreaks of Listeriosis[3].

The genus *Listeria* is included among the Coryne form bacteria that contain six species: *L. monocytogenes, L. ivanovii, L. innocua, L. grayi, L. welshimeri* and *L. seeligeri*. Only *L. monocytogenes* and *L. ivanovii* are pathogenic. *L. monocytogenes* infect both humans and animals, while *L. ivanovii* is principally ad animal pathogen that rarely occurs in humans[4].

The different conditions in which *Listeria* survive in environment, the remarkable resistance in the processing area, the multiplication capacity at refrigeration temperature, the long persistence in food even in hostile conditions, make from *L. monocytogenes* an important threat for the population health status [5].

Listeriosis is of great health importance where it is seen primarily in pregnant women, newborns, elderly and immuno suppressed persons. The incidence of *L. monocytogenes* in human is relatively low, while the mortality rate is 30%. This bacterium causes intra-uterine infection, meningitis and encephalitis and septicemia. In pregnant women may result in spontaneous abortion or stillbirths. *Listeria* also causes wide range of infection including gastroenteritis, bacteraemia, pneumonia, osteomyelitis and endocardits[5].

According to many authors, *L. monocytogenes* is most commonly isolated from raw milk sampled from collection tanks and various contamination degrees have been recorded [3-5].

Therefore The aim of this work is to investigate the prevalence of *Listeria monocytogenes* and other *Listeria* spp. in raw milk and white soft cheese by conventional methods and polymerase chain reaction.

METHODOLOGY

This study was carried out in the Microbiology and Immunology Department, Faculty of Medicine, Mansoura University during the period form October 2015 to May 2016.

Samples:

Two hundreds Samples (50 of each market raw milk, individual farm milk and Kariesh cheese plus 25 of each Damietta cheese and Talaga cheese) were randomly collected form dairy farms, different shops and super markets in Mansoura city, Dakahlia governorate. Egypt.

Raw market milk and individual farm milk samples were represented by 500 ml, while cheese samples were represented by 250 gm. All samples were aseptically collected in sterile plastic bags or sterile capped bottles and kept in an icebox containing ice packs and transported immediately to the Microbiology laboratory where they were analysed at once.

Bacteriological examination:

Preparation of samples:

Milk Samples:

Each milk sample was agitated, then 25 ml of samples were aseptically add to 225 ml of tryptone soya broth and mixed well.

Cheese Samples:

Each cheese sample must be representative of outer and interior part, then 25 gm of sample was aseptically added to 225 ml of tryptone soya broth in sterile blender, and then homogenized thoroughly.

Isolation of *Listeria* Spp.

Direct plating technique:

100 µl of each prepared homogenate was streaked onto Oxford agar plates (CM85b, Oxoid) supplemented with *Listeria* selective supplement (cycloheximide, colistin sulphate, acriflavin, cefotetan and fosfomycin) (SR140, Oxoid) by using sterile glass rod for spreading the inoculum onto the plates. Then Plates were incubated at 37ºC for 48 h and examined for suspected colonies (gray green colonies surrounded by black zone of esculine hydrolysis). At least five presumptive colonies from each chosen plate were picked up and subcultured onto tryptone soya agar slants (CM131, Oxoid). All slants were incubated at 37 ºC for 24 h, then subjected to biochemical identification.

Enrichment technique:

The prepared homogenate was incubated at 37 ºC for 24 h, then 10 ml of incubated homogenate was added to second enrichment of 90 ml of Listeria enrichment broth base with Listeria selective enrichment supplement (Nalidixic acid, cycloheximide, Acriflavin hydrochloride) (SR141, Oxoid) and incubated at 37 ºC for 48 h. After that, 100 ul from second enrichment broth was streaked onto Oxford agar plates (CM85b, Oxoid) supplemented with Listeria selective supplement (cycloheximide, colistin sulphate, acriflavin, cefotetan and fosfomycin) (SR140, Oxoid) by using sterile glass rod for spreading the inoculum onto the plates which were incubated at 37 ºC for 48 h. Incubated plates were examined for suspected colonies (gray green colonies surrounded by black zone of esculin hydrolysis). At least five presumptive colonies from each selective plate were picked up and subcultured onto tryptone soys agar slants (CM131, Oxoid) and incubated at 37 ºC for 24 h, then subjected to biochemical identification[6].

Identification of Listeria spp.:

Microscopic examination:

By using Gram stain, films were prepared from the pure culture of isolated orgnisms and examined microscopically for the presence of Gram positive small rods which sometimes arranged in short chains[6].

Biochemical identification:

Suspected colonies from the pure culture of isolated organisms were subjected to the following tests:
motility test, oxidase test, catalase test, CAMP test, rhamnose fermentation test, xylose fermentation test and haemolysis on 7% sheep blood agar. *Listeria* spp. was identified as being motile, oxidase-negative, catalase – positive, giving positive CAMP test with *Staphylococcus aureus*, ferments rhamnose with no gas but did not ferment xylose and may show haemolysis on blood agar[6]. The isolates were further identified to species level by 10300 API Listeria strips (bio Mérieux, Durham NC, USA).

Molecular examination:

Detection of *Listeria monocytogenes* hylA gene by colony PCR:

Listeria monocytogenes isolates were screened for the presence of Listeriolysin O (hyl A). The sequence of the oligonculeotide primers was: Forward hyl A 634 F: 5'-ACTTCGGCGCAAT CAGTGA-3 AND Reverse hylA 770R: 5'-TTGCA ACTG CTCTTTA GT AACAGCTT-3' [7].

DNA extraction:

The extraction of DNA was done by picking up 1-2 colonies of suspected isolates by sterile tips and suspended in 50 ul distilled water and subjected to heat block at 95ºC for 10 min[7].

PCR reaction:

PCR was performed in a total reaction of 25 µl, with mixture of 12.5 µl of Dreem Tag Green PCR Master Mix (Fermentas), 1µl forward primer, 1µl reverse primer, 9.5 µl RNAs free water and 1µl from DNA template. Cycling conditions were: initial denaturation at 95 ºC for 5 min, followed by 40 cycles consisting of 95 ºC for 30s, 60 ºC for 30s and 72 ºC for 1 min with final extension at 72 ºC for 5 min. PCR products were then visualized using ethidium bromide stained 1.5% agarose gel electrophoresis. The separated products were then visualized under UV light and photographed. *Listeria monocytogenes* hyl A gene was detected at 136 bp.

RESULTS

By studying the prevalence of *Listeria* spp. in milk and white soft cheese by direct plating method, it has been showed that *Listeria seeligeri* revealed highly significant variation at Damietta cheese, form milk and Kariesh cheese. On the other hand, *Listeria ivanovii* showed highly significant variation at Talaga cheese while *Listeria monocytogenes* gave highly significant variation at raw market milk. Table (1).

Table 1: Prevalence of *Listeria* spp. In milk and white soft cheese by direct platting method.

Isolates / Milk & dairy products	Total no. of samples	No. of positive samples		*Listeria monocytogenes*		*Listeria ivanovii*		*Listeria seeligeri*	
Raw milk		**No. of samples**	**%**	**No. of samples**	**%**	**No. of samples**	**%**	**No. of samples**	**%**
1) Raw market milk	50	16	32	5	10	12	24	6	12
2) Individual farm milk	50	21	42	ND	0	12	24	15	30
White soft cheese									
3) Acid curd Kariesh cheese	50	24	48	4	8	8	16	16	32
4) Fresh soft cheese (Talaga)	25	16	64	2	8	12	48	9	36
5) Damietta cheese	25	13	52	1	4	6	24	9	36
Total	200	90	45	12	6	50	25	55	27.5

ND: not detected

By enrichment method, the prevalence of *Listeria* spp. in milk and white soft cheese should that *Listeria seeligeri* gave highly significant variation at farm milk and both *Listeria ivanovii* and *Listeria monocytogenes* revealed highly significant variation at raw market milk (Table 2).

Table 2: Prevalence of *Listeria* spp. In milk and white soft cheese by enrichment method.

Isolates / Milk & dairy products	Total no. of samples	No. of positive samples		*Listeria monocytogenes*		*Listeria ivanovii*		*Listeria seeligeri*	
Raw milk		**No. of samples**	**%**	**No. of samples**	**%**	**No. of samples**	**%**	**No. of samples**	**%**
1) Raw market milk	50	7	14	4	8	7	14	1	2
2) Individual farm milk	50	8	16	ND	0	3	6	7	14
White soft cheese									
3) Acid curd Kariesh cheese	50	2	4	ND	0	ND	0	2	4
4) Fresh soft cheese (Talaga)	25	6	24	1	4	3	12	3	12
5) Damietta cheese	25	5	20	ND	0	3	12	4	16
Total	200	28	14	5	2.5	16	8	17	8.5

ND: not detected

The distribution of different *Listeria* spp. isolates in milk and white soft cheese revealed that *Listeria ivanovii* showed highly significant variation at both raw market milk and Talaga cheese, while *Listeria seeligeri* gave high significant variation at farm milk, Kariesh cheese and Damietta cheese. *Listeria monocytogenes*, on the other hand, revealed highly significant variation at raw market milk (Table 3).

Table 3. Total Distribution of *Listeria* spp. isolates in milk and white soft cheese.

Isolates / Milk & dairy products	Total no. of samples	*Listeria monocytogenes*		*Listeria ivanovii*		*Listeria seeligeri*	
Raw milk		**No. of isolates**	**%**	**No. of isolates**	**%**	**No. of isolates**	**%**
1) Raw market milk	58	19	32.7	31	53.5	8	13.8
2) Individual farm milk	53	ND	0	22	41.5	31	58.49
White soft cheese							
3) Acid curd Kariesh cheese	44	5	11.36	15	34	24	54.5
4) Fresh soft cheese (Talaga)	55	4	7.27	31	56.36	20	36.36
5) Damietta cheese	39	1	2.5	11	28.2	27	69.23
Total	249	29	11.64	110	44.17	110	44.17

ND: not detected

By comparing the detection rate of *Listeria monocytogenes* by conventional methods and PCR in different samples, the conventional methods detected 29 (14.5%) of 200 samples examined versus 31 (15.5%) detected by PCR with no significant difference. (Table 4, Figure 1).

Table 4: Detection rates of *Listeria monocytogenes* by conventional methods and PCR:

Isolates / Milk & dairy products	Number. of samples	*Listeria monocytogenes*			
		Conventional methods		**PCR**	
Raw milk		**No. of isolates**	**%**	**No. of isolates**	**%**
1) Raw market milk	50	19	38	20	40
2) Individual farm milk	50	ND	0	1	2
White soft cheese					
3) Acid curd Kariesh cheese	50	5	10	5	10
4) Fresh soft cheese (Talaga)	25	4	16	4	16
5) Damietta cheese	25	1	4	1	4
Total	200	29	14.5	31	15.5

ND: not detected

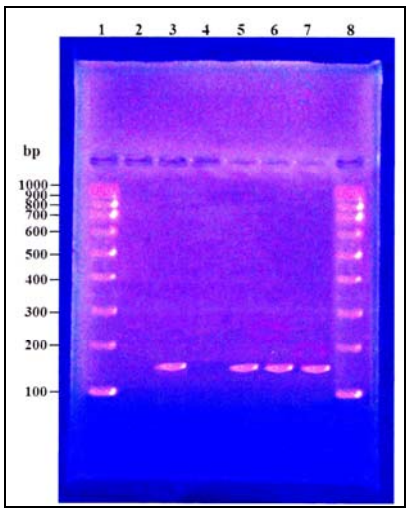

Fig. (1). Agarose gel electrophoresis of hylA gene amplicons specific for *Listeria monocytogenes*

Lane 1, 8: 100-bp DNA ladder
Lane 2, 4: Negative control.
Lane 3,5 : Samples positive for (136) base pair segment in hylA gene of *Listeria monocytogenes*
Lane 6,7 : Positive control.

DISCUSSION

Listeria monocytogenes is a food-borne pathogen of great concern for the food producing companies. Due to its physiological characteristics, such as resistance to acidic and sodium chloride stress, ability to grow at low temperature and ability to form biofilm, it can persist and/or re-contaminate food products, thereby representing an important risk for the safety of the consumers[8,9].

Raw milk can be contaminated with *Listeria monocytogenes* by many ways as poor quality of silage, inadequate frequency of cleaning the exercise area, poor cow cleanliness, insufficient lighting of milking brans and parlors and incorrect disinfection of towels between milkings[10,11].

Listeria monocytogenes is the causative agent of listeriosis, a severe infectious food-borne disease characterized by high fatality rate compared with those of other food-borne bacteria[12]. Outbreaks of human listeriosis linked to dairy products consumption have been recorded by many authors[13,14].

In the present study, Oxoford agar was used as selective medium for *Listeria monocytogenes* isolation

and identification where this medium is still the pereferred standard selective one[15]. *Listeria* spp. can be differentiated by haemolysis where *Listeria monocytogenes*, *Listeria ivanovii* and *Listeria seeligeri* demonstrate β-haemolysis[16]. However, in this study 6 out of 29 isolates of *Listeria monocytogenes* showed α-haemolysis. In order to improve the assessment of haemolysis, the CAMP test recommended to use β-haemolysis producing *Staphylococcus aureus* strain streaked in sheep blood agar[6].

Direct isolation of *Listeria monocytogenes* in this study revealed that its prevalence in raw market milk and farm milk was 10% and 0%, respectively. On the other hand, enrichment isolation of *Listeria monocytogenes* showed that its prevalence in raw market milk and farm milk was 8% and 0%, respectively. This revealed that direct isolation of *Listeria monocytogenes* was high compared with enrichment method. This can be explained that some Listeria positive samples may be undetected due to overgrowth by other Listeria spp. and/or natural flora during enrichment[17].

These findings agreed with that of other investigators who detected *Listeria monocytogenes* by direct isolation in 12% of samples and by enrichment isolation in 10% only[18,19].

Soft cheeses provide appropriate growth conditions for *Listeria* not only because of psychrotropic and halotolerent nature of *Listeria*, but also because they are commonly consumed without cooking or heating to decrease contamination during ripening period. Presence of *Listeria monocytogenes* in cheese differ according to the type of cheese, the manufacturing, inadequate pasteurization, post-pasteurization contamination, ability to multiply during storage at low temperature and resistance to sanitation[20,21].

In this work, the prevalence of *Listeria monocytogenes* detected by direct method was 8%, 8% and 4% in Kariesh cheese, Talaga cheese and Damietta cheese, respectively. On the other hand, by enrichment method, *Listeria monocytogenes* was found only in 4% of fresh soft Talaga cheese and failed to be detected in Kariesh cheese and Damietta cheese.

Many workers compared selective enrichment and non selective enrichment methods for detection of *Listeria monocytogenes*. They stated that completely selective enrichment procedure was not always the best choice for the detection of stressed *Listeria monocytogenes* in cheeses. The methods that incorporated a nonselective enrichment step gave better results than the completely selective method[22].

These results were matched with that of other authors who isolated *Listeria monocytogenes* from 3.57% of brining maturated cheese[23]. While others estimated *Listeria monocytogenes* from 3.9% of soft cheese [24]. These results were lower than that obtained by other investigators who detected *Listeria monocytogenes* in 11.1% of brined white cheese sold in Jordon[25].

Other workers reported high prevalence of *Listeria monocytogenes* in 13.6% of cheese samples[26].

A lower prevalence of *Listeria monocytogenes* contamination in cheese was noticed compared with raw milk. This may by due to the acidic nature by lactic acid bacteria which inhibit growth of *Listeria monocytogenes* in cheese[27].

The highest prevalence of *Listeria monocytogenes* in milk than in cheese showed that the physiochemical and microbiological characteristics of lactic cheeses caused a decrease of *Listeria monocytogenes* counts. However, this decrease did not lead to complete disappearance of the pathogen and *Listeria monocytogenes* was able to survive in soft lactic cheeses[28].

To eliminate the enrichment culturing step, PCR-based detection systems, which are specific and sensitive, have been proposed[14]. However, application of PCR for direct detection of pathogens present in foods has been limited by the complex consumptions of the starting materials, which contain inhibitors for PCR amplification[29,30].

RECOMMENDATION

From the results of this study and that of other workers, it could suggest that the general principles of food hygiene should still be enforced in order to minimize count of *Listeria monocytogenes* in milk and dairy products during handling, storage and manufacturing in traditional dairies. Control of the feeding cattle and milk pasteurization can also limit the contamination with *Listeria monocytogenes*.

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