Studies On Occurrence And Heat Treatment Of Listeria Monocytogenes In Milk And Some Dairy Products

***Saleh, M.A. Moustafa; *Enas M. Samy and **M.K. Rizk**
Animal Health Research Institute (Zagazig* and Mansoura** Provincial Lab.)

ABSTRACT

A total of 210 random samples of 2 groups, the first group contain 120 samples of raw milk, water, animal feeds and animal feces (30 samples of each type) and second group contain 90 samples of sterilized milk, kareish cheese and yoghurt (30 samples of each type) were collected from some private farms in Sharkia Province, samples examined for the presence of Listeria species. Three Listeria spp. were isolated from raw milk, water, animal feeds, animal feces, sterilized milk, kareish cheese and yoghurt; *L. monocytogenes* was presented by 3.3%, 0%, 6.6%, 3.3%, 3.3%, 3.3% and 0% ; *L. innocua* was present by 3.3%, 0%, 10%, 10%, 0%, 3.3% and 0%; from raw milk, water, animal feeds, animal feces, sterilized milk, kareish cheese and yoghurt respectively and *L. welshimeri* was present by 0% from raw milk, water, animal feeds, animal feces, sterilized milk, kareish cheese and yoghurt respectively. The present investigation showed three trials on sterile milk which were inoculated with 4 x 10^8 CFU/ml of *L. monocytogenes* as an initial count to detect the thermal resistance of the organisms when exposed to boiling temperature (60°C) at different times(1-4 minutes). The obtained results of the three trials revealed that the number of the organisms decreased from 4 x 10^8 to 2.96 x 10^2, 2.9 x 10^2 and 2.72 x 10^2 for the first, second and third trial. while at the fourth minute from beginning of the boiling the organisms could not be detected. By using antibiotics sensitivity testing indicated that danofloxacin, enrofloxacin and norfloxacin were the most effective antibiotics, while erythromycin was not effective. The public health importance and the sanitary measures for controlling of these organisms were discussed.

INTRODUCTION

Bacteria of genus Listeria are widely distributed in the environment, in soil, water, rotting vegetables and faeces of human and animals *(1)*. *L. monocytogenes* is a pathogenic species causing Listeriosis, incidence of *L. monocytogenes* has been reported in the last two decades. Food products frequently associated with Listeriosis specially food containing meat and dairy products *(2)*.

L. monocytogenes is a Gram-positive, motile, rode shaped, non sporulating intracellular bacterium that was able to replicates inside infected host cells. Infections occur world wide in various animals and human. It may be fatal in immunocompromised individuals, such as elderly, pregnant women, newborns and diabetic patients *(3)*.

L. monocytogenes can grow over a wide range of temperature (- 0.4 to 50°C) which coupled with pathogenecity, it makes this microorganism a potential hazard in food products *(4)*. It plays an important role as a causative agent of abortion in both human and animals, abscess in liver, arthritis, peritionities, endocarditis, conjunctivitis, pneumonia, septicemia and meningeo-encephalitis. Many other clinical forms have been published by different workers including mastitis *(5)*.

Detection and characterization of *L. monocytogenes* play an important role in controlling food borne human listeriosis. Since, the traditional methods for the recovery and identification of this pathogen in food are both labor-intensive and time consuming.

Determination of the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of an antimicrobial agents against infectious agents is more frequently requested because some bacterial infections may require treatment with bactericidal rather than bacteriostatic antibacterial *(6)*.

Due to increase interest of Listeria spp. particularly *L. monocytogenes* as health risk affecting both human and animal, therefore, this study was undertaken to determine the contamination of milk and milk products by *L. monocytogenes* and study the effect of heat in reduction of the population of *L. monocytogenes* in milk and milk products.

MATERIAL AND METHODS

Collection of samples

One hundred and twenty samples of raw farm milk, water, feeds and feces (30 samples of each) were collected randomly from private farms in Sharkia Province. Samples were collected in clean, dry and sterilized containers, then transferred rapidly as soon as possible to laboratory for microbiological examination.

Ninety samples of sterilized milk, kareish cheese and yoghurt (30 samples of each) were collected from different supermarkets and localities in Sharkia Province. Samples were kept in refrigerator till examined microbiologically.

Isolation of Listeria spp.

Twenty five grams of each sample were added to 225 ml. sterile peptone water and mixed before incubation at 30°C for 24 hours *(7)* (primary enrichment media). A portion of 0.1 ml. from the primary enrichment was transferred to tube containing 10 ml. Fraser broth for secondary enrichment and incubated at 35°C – 37°C for 48 hours. Loopful of secondary enrichment broth was streaked on a plate of PALCAM (Listeria selective medium) and inoculated plates were incubated at 35°C – 37°C for 48 hours.

Identification

Colonies suspected to *be L. monocytogenes* were identified *(8, 9)* and characterized by Gram stain *(10),* tumbling motility, Voges Proskour, catalase, oxidase, haemolysis on horse blood agar and CAMP test, for further confirmation of *L. monocytogenes*, the isolates were inoculated into 10% aqueous stock solution of Manitol, *L. rhamnose* and D. Xylose *(11)*.

Bio-typing

Identification of Listeria spp. was carried out by the Commercial Biochemical API Listeria tests (Bio Merieux Mercy I' Eloile, France) and synergestic hemolysis with *Staph. aureus* and *Rhodococcus equi* by CAMP's test *(12, 13)*. It was differentiated from other Listeria species by the analyl peptide hydrolysis test *(14)*.

Sero-typing

The study was performed on the isolated *L. monocytohenes* with Commercial Listeria O antisera (Denka, Seiken, Tokyo, Japan) as described by the manufacturer using the heating at 80°C for 1 hour of washed cell suspension in phosphate buffered saline (PBS – pH, 7.2).

Enumeration of Listeria monocytogenes

L. monocytogenes count was determined as follows: a) a single of 25gm or 25ml was removed and transferred to a bag containing 225ml of 2% sodium citrate solutions, b) homogenization for 2 minutes at room temperature in stomacher, c) each sample was serially diluted in 0.1% sterile peptone water and a total of 333µl of the diluted samples was spread plated onto three modified Oxford agar plates, d) the plates were incubated for 48 hours at 35°C *(15)*.

Antibiotyping

L. monocytogenes isolates were inoculated in brain heart infusion agar (BHI) (Difco, Detroilm USA). Susceptibility to antibacterial were done on Muller-Henton broth (MHB-Diagnostic Pasteur, France) to determine the patterns of MICs *(16)*. The following antibiotics were provided by their manufactures, Ampicillin (Beecham-Research Lab., UK); Cepheradine (Bristol-Myers Squibb Co. New York), Tetracycline (Roussel, Paris); Gentamycin (Elililly, USA); Neomycin (Upjohn); Chloramphnicol (Roussel, Paris); Nalidixic acid (Sterling Winthrop AB, Sweden); Norfloxacin (Merck Co. Inc. Rahway, NT); Trimethoprim (Roche, Paris) and Cefotaxim, streptomycin (Roche, Paris).

Culture preparation

L. monocytogenes strain was obtained from Institute of Milk Hygiene and Technology, Vet. Med. Univ., Vienna, Asstria. The strain was inoculated into Listeral Enrichment broth followed by plating 0.1ml from decimial dilution onto PALCAM agar. The inoculation was done at 27°C for 48 hours for typical colonial morphology and purity.

Effect of heat treatment (boiling) on survival of *L. monocytogens*

Experimental procedure

The previous strain was inoculated in sterile milk tested to ensure its freedom from *L. monocytogenes* to provide 4 x 10^8 CFU/ml. Several trials were designed to confirm the effect of time (1-4 minutes) colony forming unit was dected every minutes after eaching boiling. *L. monocytogenes* and count of it was obtained by direct plating of decimal dilutions of the boiling inoculated milk ***(15)*** into PALCAM agar plates which incubated at 27°C for 14 – 48 hours and typical colonies presumed to be *L. monocytogenes* were counted throught 4 minutes .

RESULTS AND DICUSSION

The recorded data in Table 1 showed the rate of Listeria species isolated from examined samples on PALCAM agar, where only 2/3 (6.6%) of raw milk, 0/30 (0%) from drinking water source, 5/30 (16%) from animals feed were positive for presence of Listeria species. Listeria species were detected in raw milk at percentage ranged from 2 – 6% ***(17-22)*** whereas higher percentages of isolation rates 12 – 23% wrere recorded ***(23-25)*** , whereas *L. Monocytogen* failed to be detected from bulk tank milk ***(26)***.

Due to good filtration and addition of some chemicals as alum, Listeria spp. could not be detected in examined water samples.

In Egypt, animal feeds including hay and concentrates were found to be major sources of Listeria species. In our study, Listeria species were isolated from 5/30 (16.6%) of examined feed samples (Table 2), this agreement with ***(27)*** who reported that the of presence of either pathogenic and non pathogenic species of Listeria in animal feeds whereas ***(28)*** reported that approximately 82% of the feed samples were harbourd Listeria spp.

Table 3 showed biochemical identification and serotyping of Listeria spp., suspected Listeria isolates were obtained from PALCAM agar positive plates of raw milk, feeds, fecal samples, sterilized milk and kareish cheese.

The presence of *L. monocytogenes* in the previous samples was 3.3%, 0%, 6.6%, 3.3%, 3.3%, 3.3% and 0%, respectively.

The presence of *L. innocua* was 3.3%, 0%, 10%, 10%, 0%, 3.3% and 0% respectively. Nearly similar results were stated by several investigators ***(29-32).***

On the other hand *L. monocytogenes* could not detect in raw milk ***(22, 33)***.

El-Kholy and El-Leboudy,(19) isolated *L. monocytogenes* in low percentage about 2% from milk of cows, whereas the presence of *L. monocytogenes* was detected in higher percent (13%, 9.5% and 22.2%) in raw milk of cows ***(23, 24, 34)***.

Unnerstad et al.,(35) isolated *L. monocytogenes* and *L. innocua* by 6% and 2% respectively from dairy cows feacal samples.

From the results recorded in Tables 2 and 4 it is obvious that out of 30 sterilized milk samples examined only one sample (3.3%) was found to be positive for *L. monocytogenes* . In 1995 an outbreaks of listeriosis due to consumption of improper sterilized and flavoured sterilized milk was recorded in the United States ***(36,37),*** also 49 cases of Listeriosis were recorded ***(38)*** due to consumption of sterilized milk in USA (1983). ***Gitter et al.,(39)*** suggested that pasteurization did not offer a quarantee of complete safety if the viable bacterial counts were high before heat treatment. However, preheating of raw milk before sterilization or pasteurization process leads to an increase heat resistance of organisms as compared with milk control ***(40, 41)***.

Table 1. Isolation rates of Listeria species in the different examined collected samples

Examined samples	*No. of examined samples*	*Positive No.*	*%*
Raw farm milk	30	2	6.6
Water	30	-	0
Animal feeds	30	5	16.6
Animal feces	30	4	13.3

Table 2. Isolation rates of Listeria species from milk products

Examined samples	*No. of examined samples*	*Positive No.*	*%*
Sterilized milk	30	1	3.3
Kareish cheese	30	2	6.6
Yoghurt	30	-	0

Table 3. Serotyping of isolated Listeria species (n = 30)

Examined samples	*L. monocytogen*			*L. innocua*			*L. welshimeri*		
	No. of isolates	+ve	%	No. of isolates	+ve	%	No. of isolates	+ve No.	%
Raw farm milk	2	1	3.3	2	1	3.3	-	-	0
Water	-	-	0	-	-	0	-	-	0
Animal feeds	5	2	6.6	5	3	10	-	-	0
Animal feces	4	1	3.3	4	3	10	-	-	0
Sterilized milk	1	1	3.3	-	-	0	-	-	0
Kareish cheese	2	1	3.3	2	1	3.3	-	-	0
Yoghurt	-	-	0	-	-	0	-	-	0

Table 4. Incidence of *L. monocytogenes* in the examined samples

Examined samples	*No. of examined samples*	*Positive No.*	*%*
Raw farm milk	30	1	3.3
Water	30	-	0
Animal feeds	30	2	6.6
Animal feces	30	1	3.3
Sterilized milk	30	1	3.3
Kareish cheese	30	1	3.3
Yoghurt	30	-	0

On the other side, the organism could not be detect in sterilized milk *(42)*.

Concerning the results in Tables 2 and 4, it is evident that out of the 30 kareish cheese samples examined, only one sample (3.3%) was found positive for *L. monocytogenes*. Nearly similar findings were previously obtained *(43, 44)*. While higher incidence of the organism in kareish cheese, which was identified from 16.6% of examined kareish cheese samples *(45)*. The existence of *L. monocytogenes* in kareish cheese could be attributed to the fact; it is usually made from raw milk, high pH value, high moisture content, the primitive way of processing, handling and methods of selling of cheese.

In case of yoghurt samples, *L. monocytogenes* or other Listeria spp. could not be detected (as in Tables 2 and 4), this finding goes parallel with the results achieved by several inestigators *(12, 46, 20, 47)*. The failure of these microorganisms to grow could be attributed to lactic acid production and the resultant lower pH value. ***Ahmed, (48)*** reported that *L. monocytogenes* could only grow at pH values ranged from 5.6 to 9.5 with optimal growth occurring at neutral to slightly alkaline pH values.

Furthermore, it is possible to state that in most of *L. monoytogenes* strains isolated from specimens and products with a biochemical of non-hemolytic *L. monocytogenes* on blood agar serotype 4 ab are not *L. monocytogenes*, but a non-pathogenic *L. innocua*, this is very important from the point of view of evaluation of the followings *(49)*.

The drug susceptibility is one of the important factors of characterization of *L. monocytogenes*. Antibiotics sensitivity testing indicated that danofloxacin, enrofloxacin, and norfloxacin were the most effective antibiotics, while erythomycine was not effective. The other used antibiotics showed different degrees of antimicrobial sensitivity reactions (Table 5). ***Ibrahim and Hassan (50)*** showed that chloramphinicol and norfloxacin are considered as the antibiotic of choice. The results of tube agglutination technique revealed that danofloxacin, enrofloxacin and norfloxacin were highly effective in inhibiting bacterial growth[51] isolated from raw milk at low concentrations (0.022 – 0.048 ug/mL). Determination of MICs and MBCs reflected that these drugs possess bactericidal effect against tested bacteria. These drugs inhibited the bacteria DNA synthesis at concentrations similar to those inhibited the growth of organisms.

Results from the second part of the study indicated in Table 6 depicted the effect of boiling on the growth and survival of *L. monocytogenes* ; from the tabulated data The obtained results of the three trials revealed that the number of the organisms decreased from 4×10^8 for reaching 2.96×10^2, 2.9×10^2 and 2.72×10^2 for the first, second and third trial whereas reaching 2.86×10^2 constitute the average of three trials at third minute from reaching degree of boiling (60°C), while at the fourth minute from beginning of the boiling the organisms could not be detected. The organisms failed to be detected and completely disappeared after the fourth minute. *L. monocytogenes* died rapidly in milk heated at 80°C *(52)*, while it was rapidly inactivated in milk at 62°C *(53, 54)*.

On other hand, the obtained results were in disagreement with *(55-57)*. The heat resistance of *L. monocytogenes* may be due to the intracellular state of organism in naturally contaminated milk *(36)*.

It is noteworthy from these trials that the reduction in viable cell number of *L. monocytogenes* and loss in its viability may be due to the thermal processing which is considered the most widely used method to preserve food.

In general from the public health point of view, application of good hygienic measures during production, handling and filling in final containers is essential to safe the quality of milk and its products. Consequently prevent the risk of human hazards. In addition, it is important for good hygienists and employers working in the field of dairy production to understand the pattern of microbial growth specially those of public health concern as *L. monocytogenes* to safeguard human health.

Table 5. Minimal inhibitory concentrations (MICs) and minimal bacterial concentrations (MBCs) of some antimicrobial agents against *L. monocytogenes* isolates (ug/mL)

Examined samples / Antimicrobial	Sterilized milk		Kareish cheese		Yoghurt	
	MIC	MBC	MIC	MBC	MIC	MBC
Danofloxacin	0.022	0.022	0.042	0.038	0.052	0.064
Enrofloxacin	0.024	0.048	0.044	0.062	0.034	0.052
Norfloxacin	0.048	0.056	0.046	0.085	0.050	0.013
Doxycycline	0.12	0.062	0.18	0.073	0.172	0.096
Erythromycin	2.5	3.24	2.32	3.46	2.73	3.38
Nalidixic acid	0.23	0.092	0.31	0.097	0.19	0.081
Chloramphnicol	0.16	0.059	0.18	0.062	0.22	0.042
Tetracycline	0.052	0.058	0.048	0.060	0.074	0.086
Ampicillin	0.39	0.39	0.44	0.68	0.26	0.108
Cepheradine	0.162	0.088	0.252	0.071	0.146	0.0175
Neomycine	0.082	0.96	0.079	0.98	0.062	0.094
Gentamycin	1.56	1.56	0.92	0.185	0.99	0.82
Trimethoprim	0.38	0.054	0.26	0.067	0.16	0.043
Cefotaxim	0.32	1.26	0.22	0.98	1.02	1.04

Table 6. Effect of heat treatment (boiling) on *L. monocytogenes*

Time by minute	Colony forming unit (CFU/ml)			
	First trial	Second trial	Third trial	Average
0	4×10^8	4×10^8	4×10^8	4×10^8
1	2.82×10^7	3.2×10^6	4.1×10^5	10.6×10^6
2	2.04×10^4	2.96×10^4	3.88×10^5	1.46×10^5
3	2.96×10^2	2.9×10^2	2.72×10^2	2.86×10^2
4	0	0	0	0

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الملخص العربي

دراسات علي مدي تواجد و تأثير المعالجة الحرارية علي ميكروب الليستريا مونوسيتوجينز في اللبن وبعض منتجاته

محمد علي صالح مصطفي ، إيناس محمود سامي ، مدحت كمال رزق

أجريت هذه الدراسة لاستبيان مدى تواجد ميكروب الليستريا مونوسيتوجينز في مجموعتين من العينات، في الأولي تم تجميع ١٢٠ عينه من بعض المزارع الخاصة بمحافظة الشرقية (٣٠ عينه لكل من اللبن الخام، الماء، علف الحيوان وعينات براز) وقد أسفرت النتائج عن تواجد ميكروب الليستريا مونوسيتوجينز بنسبة ٣,٣%، ٠%، ٦,٦%، و٣,٣% علي التوالي. أما في المجموعة الثانية فقد تم تجميع ٩٠ عينه من بعض منتجات الألبان (اللبن المبستر، الجبن القريش واللبن الزبادي "٣٠ عينة من كل منتج") من بعض المحال والأسواق في محافظة الشرقية، وقد أمكن عزل ميكروب الليستريا مونوسيتوجينز بنسبة ٣,٣% في كل من عينات اللبن المبستر والجبن القريش أما عينات الزبادي فقد كانت خالية من الميكروب. كما تم دراسة التأثير الحراري علي نمو ومقاومة وفناء الميكروب من خلال تأثير درجة الغليان (٦٠°م) علي اللبن وقد أسفرت النتائج عن تناقص العدد الكلي للميكروب من ٤ × ١٠^٨ إلي ٢,٩٦ × ١٠^٢ و ٢,٩ × ١٠^٢ و ٢,٧٢ × ١٠^٢ في المحاولات الأولي والثانية والثالثة علي التوالي بعد ثلاث دقائق من بداية درجة الغليان، بينما لم يتم عزل الميكروب بعد الدقيقة الرابعة في الثلاث محاولات. تم عمل اختبار حساسية للميكروب المعزول من منتجات الألبان (اللبن المبستر، الجبن القريش واللبن الزبادي) وجد أن أفضل المضادات الحيوية تأثيراً علي الميكروب الدانوفلوكساسين ، الانروفلوكساسين والنورفلوكساسين، كما وجد أن أقل المضادات الحيوية تأثيراً هي الارثرومايسين. ولقد ناقش البحث الأهمية الصحية والإجراءات التي ينبغي إتباعها لمنع تلوث المنتجات سابقة الذكر بهذا الميكروب.