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A SURVEY ON THE PRESENCE OF LISTERIA SPECIES IN RAW MILK, ICE CREAM AND HUMAN STOOLS WITH CHARACTERIZATION OF SOME ISOLATES BY SDS-PAGE

(with 7 Tables and one Figure)

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البحث عن ميكروبات الليستيريا فى اللبن الخام والآيس كريم وبراز الإنسان مع تحديد خواص بعض العترات باستخدام SDS-PAGE

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تعتبر ميكروبات الليستيريا من الميكروبات واسعة الإنتشار فى الطبيعة ومنها الليستيريا مونوسيتوجينس الذى يسبب الإصابة بمرض Listeriosis ، من هنا كان الاهتمام بالبحث عن تواجده فى الألبان والآيس كريم لما يشكله من خطورة بالغة على صحة الانسان. لذلك تم فى هذه الدراسة جمع ٢٥٠ عينة بصورة عشوائية من اللبن الخام المباع فى الأسواق والآيس كريم بنوعيه ، المصنع بواسطة صغار المنتجين والمصانع الكبيرة ، والتى تباع فى أسواق مدينة أسيوط ، بالإضافة إلى فحص عينات من كل من ألبان المزارع وعينات من براز العاملين بتلك المزارع من القائمين على حلب ورعاية الحيوانات الحلابة، بواقع ٥٠ عينة من كل نوع ، لدراسة مدى تلوثها بهذه البكتريا وقد أسفرت النتائج عن تواجد الليستيريا بنسب ٦ ، ١٤ ، ١٤ و٦% فى كل من اللبن الخام المباع فى الأسواق ، الآيس كريم المنتج بواسطة صغار المنتجين ، لبن المزارع وبراز العاملين بهذه المزارع على التوالى. بينما كانت عينات الآيس كريم المنتجة من المصانع خالية تماما من هذه الميكروبات. وقد تم تصنيف العترات المعزولة إلى ليستيريا جرايای بنسبة ٤% من كلا اللبن الخام المباع فى الأسواق أو المزارع ، ١٠% من الآيس كريم المنتج بواسطة صغار المنتجين ، وتمثلت الليستيريا مورايای بنسبة ٢ ، ٤ ، ٦ و٢% فى اللبن الخام ، الآيس كريم المنتج بواسطة صغار المنتجين ، لبن المزارع وبراز العمال على التوالى. أما بالنسبة إلى الميكروب وحيد الخلية الليستيريا مونوسيتوجينس وأيضا الليستيريا إيفانوفى فقد كانت نسبة عزلهما ٢% لكل من لبن المزارع وبراز العاملين بها ، وتم تصنيفه إلى نوع سيرولوجى رقم O_1. ونظرا لضراوة هاتين العترتين فقد أجرى اختبار الحساسية لكل منها بإستخدام تسعة أنواع من المضادات الحيوية المختلفة. كما تمت دراسة البروتين الخاص بهذه العزلات بواسطة SDS-PAGE هذا وتمت مناقشة الأهمية الصحية والوبائية لميكروبات الليستيريا وعزلاتها المختلفة والشروط الواجب

إتباعها ، وخاصة فى مزارع الألبان من توعية للعاملين بها ، لمنع تلوث الألبان والآيس كريم بهذه الميكروبات لدرء خطرها عن المستهلكين.

SUMMARY

Listeric infections, caused by microorganisms of the genus *Listeria*, occur worldwide and in a variety of animals and man. Listeriosis was recognized as a food-borne human disease that prompted intense research activity. Thus, between April and September 2003, a total of 250 samples including raw marketable milk (50) and ice cream of both small and large scale producers (50 each) purchased from retail local markets, supermarkets and street vendors in Assiut City, as well as farm milk (50) and human stools of apparently healthy farm attendants (50) collected from dairy farms. These samples were examined to investigate the prevalence of *Listeria* species. Also, antibiotic sensitivity pattern as well as characterization of the isolated strains, by (SDS-PAGE), were performed to find out the degree of homogeneity between those isolates. *Listeria* spp could be detected in 3 (6%), 7 (14%),7 (14%) and 3 (6%) of examined raw marketable milk, small scale ice cream, farm milk and stools of attendants, respectively, while failed detection in large scale ice cream samples. *L. grayi* was identified in 2 (4%) in both raw marketable and farm milk and 5 (10%) in small scale ice cream, while *L. murrayi* was isolated in percentages of 2 , 4 , 6 and 2% from tested samples of marketable milk, small scale ice cream, farm milk and stools, respectively. Concerning *L. monocytogenes*, it has been recovered from only one (2%) sample of each of farm milk and attendant stool, which obtained from the same dairy farm. The serotyping of these pathogens revealed that isolates were of serotype O_1. Likewise, *L .ivanovii* was detected in one (2%) of each of farm milk and attendant stool samples. Furthermore, the antimicrobial susceptibility testing of the previous strains showed that they were susceptible to the used antibiotics in percentages ranged from 88.9-100%. Also, to study the degree of homogeneity between these strains, whole cell proteins of these strains were analysed by using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

***Key words**: Listeria species, Raw milk, Ice cream, Human stool, Antimicrobial susceptibility, SDS-PAGE.*

INTRODUCTION

Listeria monocytogenes is an ubiquitous food-borne Gram +ve bacterium, responsible for life threatening infections in animals and humans with a mortality rate of 25-30% (Farber & Peterkin, 1991 and Aguado *et al.*, 1999). The genus *Listeria* currently includes seven species: *L. monocytogenes, L. ivanovii, L. seeligeri, L. innocua, L. welshimeri, L. grayi* and *L. murrayi* (Rocourt & Cossart, 1997). Two of these species, *L. monocytogenes* and *L. ivanovii* are potentially pathogenic (Vazquez-Boland *et al.*, 2001). In ruminants, *L. monocytogenes* is responsible for a variety of clinical manifestations, such as septicemia, meningitis, abortion or mastitis in dairy animals accompanied by excretion of the microorganism in milk. Moreover, this pathogen could be sheded in milk in asymptomatic animals and subsequently considered a health risk to consumers either through consumption of raw milk or milk products (Low & Donachie, 1997). Furthermore, it is believed that domesticated ruminants probably play a key role in the maintenance of *Listeria* spp. in the rural environment via a continuous fecal-oral enrichment cycle (Fenlon, 1999).

Listeriosis in human are usually associated with the hemolytic species of *Listeria* (*L. monocytogenes, L. ivanovii* and *L. seeligeri),* however, *L. welshimeri* pathogenicity to human has been well documented. (Cocolin *et al.*, 2002). *L. monocytogenes* is the only species of the genus *Listeria* that has been involved in known food-borne outbreaks of serious diseases such as septicemia, abortion, meningioencephalitis, meningitis and gastrointestinal illness in immunocompromised, elderly individual, pregnant women and their unborn or newnates (Slutsker & Schuchat, 1999; Economou *et al.*, 2000; Hof, 2001; Carrique-Mas *et al.*, 2003 and Longhi *et al.*, 2003). Furthermore, it has been reported that *Listeria* spp. could be isolated from apparently healthy persons, and these cases were attributed to exposure to high infective doses (McLauchlin *et al.*, 1990; Farber & Peterkin, 1991 and Grif *et al.*, 2001).

Listeria spp. are well equipped to survive food processing technologies. For example, they tolerate high concentration of salt and relatively low pH and worst of all, they are able to multiply at refrigeration temperatures (Lou & Yousef, 1999). Therfore, they are considered a serious threat to food safety and rank them among the microorganisms that most concern the food industry. Many surveys have been conducted and showed that *L. monocytogenes* and other *Listeria* spp. could be isolated from milk and dairy products worldwide

(Davidson *et al.*, 1989; Rohrbach *et al.*, 1992; Loncarevic *et al.*, 1995; Steele *et al.*, 1997; Yoshida *et al.*, 1998; El-Prince, 1999; Jayarao & Henning, 2001 and El-Sherbini & Abdallah, 2003). Several episodes of listeriosis were linked epidemiologically to the consumption of milk or its products where contamination takes place not only during the production of raw milk or during dairy processing but post process contamination from environmental sources has been well documented (Glass *et al.*, 1995 and Bubert *et al.*, 1999). Moreover, it has been isolated from pasteurized milk and ice cream in which contamination occurred postproduction or recontaminated during further handling (Jeong & Frank, 1994).

Therefore, this work was conducted to survey raw marketable milk, farm milk and small scale as well as large scale produced ice cream for the presence of *Listeria* spp. Moreover, stools of attendants working in dairy farms were examined to investigate whether apparently healthy human can harbor these bacteria without clinical manifestations. Also, antibiotic sensitivity pattern as well as whole cell proteins analyses by using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of the isolated strains were performed to study the degree of homogeneity between the isolated strains and to elucidate possible contamination sources, which may enable the establishment of preventive measures.

MATERIALS and METHODS

1- Collection of samples:

Two hundred and fifty random samples were collected randomly including: raw marketable milk (50), ice cream of both small and large scale producers (50 each) purchased from retail local markets, supermarkets and street vendors in Assiut City, as well as farm milk (50) and human stools of apparently healthy farm attendants (50) collected from dairy farms between April and September (2003). These samples were analyzed as soon as possible in the laboratory for the presence of *Listeria* spp. Milk samples were examined by Storch test to detect heat-treated samples according to Lampert (1975).

2- Isolation of *Listeria* spp:

Detection of *Listeria* spp. was done as described by Hitchins (1992) where, 1 ml of milk or 1g of ice cream samples as well as swabs from human stools were aseptically added to 9 ml of listeria enrichment broth (Biolife) and incubated at 35±1°C for 48 h. After incubation, a

loopful of enrichment broth was streaked on the surface of Oxford medium and incubated at 35±1°C for 24-48 h. (Curtis *et al.*, 1989).

3- Identification of *Listeria* spp:

Identification and species differentiation were carried out according to Warburton *et al.* (2003) including Gram stain, catalase test, carbohydrate fermentation, B-haemolysis on blood agar and CAMP test.

4- Serotyping of *L. monocytogenes strains*:

Biochemically identified isolates were serotyped with *listeria* O antisera type 1 (Difco) by using slide agglutination test according to Difco (1984).

5- Antimicrobial susceptibility testing for *L. monocytogenes* and *L. ivanovii*:

L. monocytogenes and *L. ivanovii* strains were tested for their sensitivity and resistance patterns to 9 different antimicrobial agents (Amoxycillin, Ampicillin, Cephalosporin, Enrofloxacin, Flumequine, Gentamycin, Lincospectin, Spectinomycin and Trimethoxin) by disc diffusion method (Harvey & Gilmour, 2001). All plates were incubated at 35± 1°C for 24 h. and examined for inhibition zones and the results were recorded.

6- Sodium Dodecyl sulphate-polyacrylamide Gel electrophoresis (SDS-PAGE):

L. monocytogenes and *L. ivanovii* strains were incubated in Brain heart infusion (BHI) broth at 37°C for 24 h. 2 ml of each culture were centrifuged at 15.000 rpm for 1 h and the pellet volume was estimated. The pellet was resuspended 1: 1 (w/v) in a sample buffer, boiled for 5 min in boiling water bath followed by spin down, and kept at -20°C until examined. 20 µl of each sample were separated by SDS-PAGE with 4% (wt/vol) and 12% (wt/vol) acrylamide for the stacking and separating gels, respectively in the discontinuous buffer system of Laemmli (Laemmli, 1970) by using a mini protean cell II (BioRad,USA) After electrophoresis the gel was stained with Commassie Blue R for 1-2 h. then the gel was destained in 40% methanol and 10% acetic acid for 1-3 h till the bands become clear and photographed.The molecular weights of the protein bands were calculated by matching the relative mobility of the protein bands and that of the prestained molecular weight marker.(Burnette, 1981).

RESULTS

Obtained results were recorded in Tables 1-7 and Figure 1.

Table 1: Isolation rate of *Listeria* spp. in the examined raw marketable milk and ice cream samples.

Types of samples	No. of tested samples	Positive samples	
		Number	%
Raw marketable milk	50	3	6%
Small scale ice cream	50	7	14%
Large scale ice cream	50	-	-

Table 2: Frequency distribution of *Listeria* spp. isolated from examined raw marketable milk and ice cream samples.

Types of samples	Isolated strains	
	No./10	%
Raw marketable milk	3	30%
Small scale ice cream	7	70%
Large scale ice cream	-	-

Table 3: Occurrence of *Listeria* spp. in the examined raw marketable milk and ice cream samples.

Types of samples	Isolated strains							
	L.monocytogenes		*L.grayi*		*L.ivanovii*		*L.murrayi*	
	No./50	%	No./50	%	No./50	%	No./50	%
Raw marketable milk	-	-	2	4%	-	-	1	2%
Small scale ice cream	-	-	5	10%	-	-	2	4%
Large scale ice cream	-	-	-	-	-	-	-	-

Table 4: Isolation rate of *Listeria* spp. in the examined farm milk and stools of attendants.

Types of samples	No. of tested samples	Positive samples	
		Number	%
* Farm milk	50	7	14%
* Human stools	50	3	6%

* Samples from the same dairy farm

Table 5: Frequency distribution of *Listeria* spp. isolated from examined farm milk and stools of attendants

Types of samples	Positive samples	
	No./10	%
* Farm milk	7	70%
* Human stools	3	30%

* Samples from the same dairy farm

Table 6: Prevalence of *Listeria* spp. in the examined samples of farm milk and stool of attendants.

Types of samples	Isolated strains							
	****L.monocytogenes**		*L.grayi*		*L.ivanovii*		*L.murrayi*	
	No./50	%	No./50	%	No./50	%	No./50	%
* Farm milk	1	2%	2	4%	1	2%	3	6%
* Human stools	1	2%	-	-	1	2%	1	2%

* Samples from the same dairy farm.
** Serotype O_1.

Table 7: Antibiotic sensitivity pattern of the isolated *L.monocytogenes* and *L.ivanovii* strains.

Types of antibiotics	Farm milk				Human stool			
	L.monocytogenes		*L.ivanovii*		*L.monocytogenes*		*L.ivanovii*	
	S	R	S	R	S	R	S	R
Amoxycillin	+		+		+		+	
Ampicillin	+		+		+		+	
Cephalosporin	+		+		+			+
Enrofloxacin	+		+		+		+	
Flumequine	+		+		+		+	
Gentamycin	+			+	+		+	
Lincospectin	+		+		+		+	
Spectinomycin	+		+		+		+	
Trimethoxin	+		+			+	+	
Total No.	9		8	1	8	1	8	1
%	100%		88.9%	11.1%	88.9%	11.1%	88.9%	11.1%

* S = Sensitive
* R = Resistant

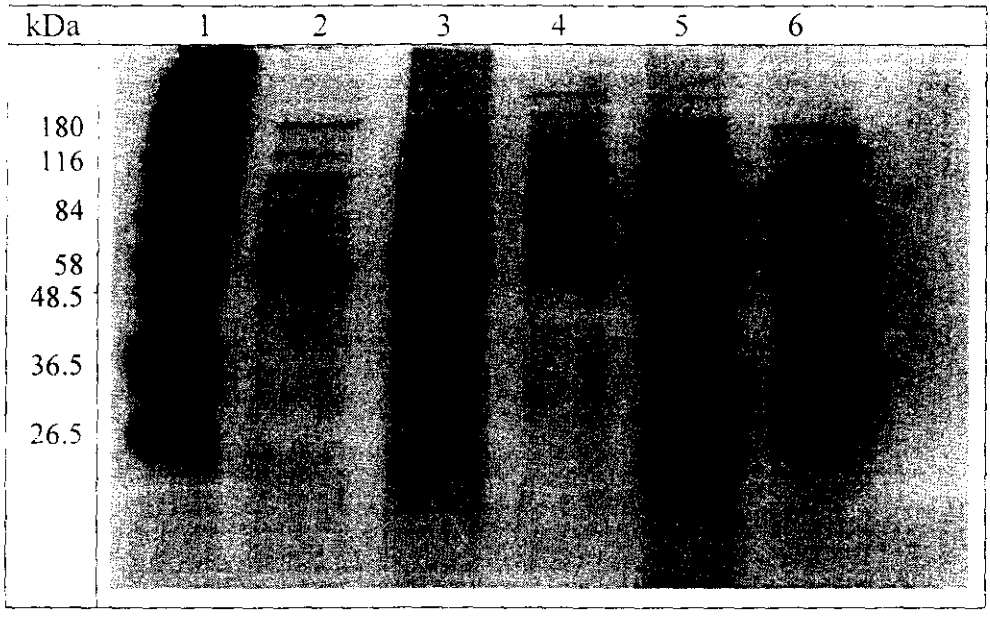

Figure 1: SDS-PAGE profiles of *L.monocytogenes* and *L.ivanovii.*
Lane : 1 : Prestained molecular weight marker (sigma). Lanes 2 and 4: strains of *L.ivanovii* isolated from farm milk and human stool, respectively. Lanes 3 and 5: strains of *L.monocytogenes* isolated form farm milk and human stool. Lane 6: *L.monocytogenes* strain obtained from Institut of Milchhygiene and Technolgie, Vet.med. Univ., Vienna, Austria.

DISCUSSION

Food-borne infection through milk and its products appears to be the major means of zoonotic transmission of listeriosis. *L.monocytogenes*, the most common causative agent of that disease, is a widespread pathogen and has been also isolated from the gastrointestinal tract of asymptomatic animals and persons as well as the environment (Glass *et al.*, 1995). Despite the low incidence of infection by this pathogen, its association with high mortality rates (25-30%) makes listeriosis a serious health problem (Aguado *et al.* 1999).

Results presented in Tables 1 & 2 declared that the isolation rate of *Listeria* spp. in the examined raw marketable milk samples was 6% with a frequency distribution of 30% of the total isolates. This incidence goes parallel with that obtained by Wahba, 2002 (6%), but it was slightly higher than those recorded by Saito *et al.*, 1991 (5.3%); Aman & Ahmed, 1997 (3.33%); Abdel-Khalek & El-Gamal, 1998 (4%) and El-Prince, 1999 (4%). On the other hand, higher findings were postulated by Harvey & Gilmour, 1992 (25%), Morales *et al.*, 1995 (7%) and

Salama, 2000 (10%). However, no *Listeria* spp. were detected in the examined 40 raw milk samples examined in the province of Bologna, Italy (Massa *et al.*, 1990). The relatively higher incidence of *Listeria* spp. could be attributed to poor sanitary measures during milk handling, production and solding. In addition, combination of enrichment procedure and selective medium employed in this survey, yielded *Listeria* strains that could not be detected by direct plating (Pednekar *et al.*, 1997).

Moreover, *Listeria* isolates from raw marketable milk were differentiated biochemically into 2 strains (4%) of *L. grayi* and one strain (2%) of *L. murrayi* as showed in Table 3. However, *L. monocytogenes* and *L. ivanovii* failed to be detected. The absence of *L. monocytogenes* agrees with results recorded by Massa *et al.* (1990), Morales *et al.* (1995) and Pednekar *et al.* (1997). While, this findings does not necessarily indicate that milk is free of risk to be infected by *L. monocytogenes* as other *Listeria* spp. were detected. Also, the physiology and habitat of the different species of *Listeria* are very similar (McLauchlin *et al.*, 1990).

In the contrary, this pathogen was previously isolated worldwide by many investigators (Davidson *et al.*, 1989; Farber & Peterkin, 1991; Rohrbach *et al.*, 1992; Loncarevic *et al.*, 1995; Steele *et al.*, 1997; Yoshida *et al.*, 1998; El-Prince, 1999, Jayarao & Henning, 2001; Wahba, 2002 and El-Sherbini & Abdallah, 2003).

In case of ice cream, it is apparent from the results outlined in Tables 1 & 2 that, *Listeria* spp. failed to be detected in the examined samples of large scale producers. While, they were recovered from 7 (14%) samples of small scale ones, with a frequency distribution of 70%. Lower incidence (7.3%) was estimated by Cotton & White (1992) however, Pednekar *et al.* (1997) recorded relatively higher results (28-42%). On the other hand, Choi *et al.* (2001) could not isolate *Listeria* spp. from any of examined ice cream samples.

Further identification showed that, *L. monocytogenes* could not be isolated from small scale ice cream samples and all the identified strains belonged to non-pathogenic species, 5 (10%) found to be *L. grayi* and 2 (4%) were *L. murrayi* (Table 3). In contrast, *L. monocytogenes* was recovered from ice cream samples examined by Cotton & White (1992); Pednekar *et al.* (1997) and Baek *et al.* (2000).The presence of other *Listeria* spp. could be attributed to unsanitary measures during handling and preparation of ice cream (Jeong & Frank, 1994). Likewise, *Listeria* spp. has the ability to survive, grow, multiply and to remain

viable in refrigerated conditions due to their psychrophilic character (Glass *et al.*, 1995).

L. monocytogenes, could be transmitted to the consumers by ingestion of contaminated milk (Low & Donachie, 1997) causing typical gastrointestinal illness (Dalton *et al.*, 1997). It is evident that 7 (14%) of farm milk samples were contaminated with *Listeria* spp. in frequency distributions of 70 % (Tables 4 & 5). Species recovered from farm milk samples were identified as 1 (2%) of both *L. monocytogenes* and *L. ivanovii*, 2(4%) of *L. grayi* and 3 (6%) of *L. murrayi* as declared in Table 6. Low & Donachie (1997) pointed out that *L. monocytogenes* and *L. ivanovii* are pathogenic, while other *Listeria* spp. are regarded as non-pathogenic.

Somewhat similar incidence of *L. monocytogenes* (1.6%) was indicated by Davidson *et al.* (1989) in raw milk in Canada. However, in USA, Lovett *et al.* (1987); Rohrbach *et al.* (1992) and Jayarao & Henning (2001) recorded higher percentages in the examined bulk milk tanks (4.2, 4.1 and 4.6%, respectively). Also, *L. ivanovii* is recorded as a cause of abortion in sheep and cattle but less frequently than *L. monocytogenes* (Alexander *et al.*, 1992).

Farm attendants considered as high-risk group to acquire or harbor pathogens due to their direct contact with animals.Our investigation revealed that 3 (6%) of stools were positive for *Listeria* spp. in frequency distribution of 30% (Tables 4 & 5) We could able to detect one strain (2%) of each of *L. monocytogenes*, *L. ivanovii* and *L. murrayi* as shown in Table 6. It has been estimated that fecal carriage rate is at least 0.8% in apparently healthy humans (Grif *et al.*, 2001). Moreover, it has been stated that isolation rate of *L. monocytogenes* from apparently healthy humans varies from 1-10% (Husu, 1990 and Farber & Peterkin, 1991). However, Dalton *et al.*, (1997) and Carrique-Mas *et al.* (2003) isolated *L. monocytogenes* from stools specimens of patients with gastroenteritis accompanied with fever with a rate of 37% and 84%, respectively.

The two isolates of *L. monocytogenes* recovered from farm milk and stool of an attendant belonged to serotype O1 (Table 6). This finding was in harmony with Dalton *et al.* (1997) in stool specimens and Yoshida *et al.* (1998) and Baek *et al.* (2000) in raw milk samples examined.

Furthermore, antibiotic susceptibility testing using nine different antibiotics in vitro, for *L. monocytogenes* and *L. ivanovii* isolates (2 each) were done as illustrated in Table 7, they were mostly susceptible to the used antimicrobial agents, however, *L. ivanovii* recovered from

farm milk, was resistant to gentamycin while, *L. monocytogenes* and *L. ivanovii* isolated from stools were resistant to trimethoxin and cephalosporin, respectively. This test found to be essential to guide the selection of antibiotic and predict the clinical response to treatment for successful eradication as well as to study the variability between the strains (Smaill, 2000).

In the present study,whole cell proteins of two strains of both *L.monocytogenes* and *L.ivanovii* isolated from farm milk and human stool were analyzed by SDS-PAGE (Figure:1). Concerning *L. monocytogenes* protein patterns of both strains of farm milk (lane 3) and human stool (lane 5) characterized by high degree of homogeneity with 16 protein bands of molecular weights (180, 162,150, 130, 122, 118, 107, 106, 105, 103, 80, 68, 58, 48, 44 and 27 kDa). The homogeneity of protein patterns between the two strains might indicate that this strain was endemic in the dairy farm and the farm attendant harboured the pathogen without any clinical symptoms which act as a risk of developing clinical gastroenteritis. Moreover the danger of contamination and cross contamination of the environment and equipments in dairy farm through the unhygienic practices done by the attendant. It was revealed that both of the isolated strains of *L.monocytogenes* were different from the examined strain of *L.monocytogenes* obtained from Institut of Milchhygiene and Technolgie, Vet.med. Univ., Vienna, Austria. which showed different protein bands with molecular weights (150, 122, 115, 105,103, 84,56,48 and 33 kDa). On the other hand protein profiles of both strains of *L.ivanovii* isolated from farm milk and human stool seems to vary from each other in their protein bands with molecular weights (130, 122, 110, 50, 48 and 36 kDa) for farm milk strain and (162, 118, 58 kDa) for human stool strain. However, both strains showed a degree of homogeneity in carrying protein bands of molecular weights (180, 130, 107, 106, 103, 68, and 58 kDa). It is concluded that both of the strains were acquired from different sources.

It is noteworthy, to state that *L. monocytogenes* is common in dairy farm environment and its main source is the feces or manure. Consequently, infected human or animals can carry this microorganism in their gastrointestinal tract even without symptoms or may retain it for several months, thus constituting potential public health hazard (Husu, 1990; Farber & Peterkin, 1991 and Fedio & Jackson, 1992). However, the low incidence of this pathogen in this investigation does not pose any practical problem for pasteurized milk and processed milk products but may be of significance for raw milk consumption (O'Donnell, 1995).

Meanwhile, pasteurization remains the first line of defense against transmission of food-borne diseases through milk or other dairy products. Fortunately, *Listeria* does not survive proper pasteurization and in food processing, Food & Drug Administrative FDA has adopted a policy of "zero tolerance" for presence of *Listeria*, in response, has introduced HACCP (Dalton *et al.* 1997 and Harvey & Gilmour, 2001). In the meantime, control of listeriosis could be achieved by awareness of the ubiquity of these pathogens and especially of those, environments that favor their multiplication. Thus, the collaboration of medical, veterinary and milk hygienists, farmers, food manufacturers and technologists, is essential.

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