Isolation and identification of *Listeria moncytogenes* by PCR *from* some food sources in Erbil city

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ABSTRACT :

The present study was carried out to evaluate foods in local markets of Erbil and Koya cities; 225 food samples were examined for the presence of Listeria species particularly *L. monocytogenes* from October 2010 to March 2011. The studied samples included 70 samples of raw poultry meat, 55 samples of raw milk, 50 samples of cheese and 50 samples of raw red meat. According to motility test, hemolysin production , sugar fermentation test, and PCR technique by using specific primer, 8 isolates of *L. monocytogenes* were identified, 1 (2%) isolate from cheese , 2 (4%) isolates from red meat and 5 (7.3%) isolates from chicken meat. The percentage of contaminated foods for Listeria species from chicken meat, raw milk, cheese and red meat were 20, 9, 20 and 22% respectively. The ability of *L. monocytogenes* to produce some enzymes which have role in pathogenicity of the bacterium like catalse, hippurate, hemolysin, protease, lipase , lecithinase, DNase, esterase, were determined, generally it was found that all the 8 isolates of *L. monocytogenes* were (100%) positive for catalase and hippurate hydrolysis, 6 isolates (75%) produce DNase and hemolysin ,7 isolates (87%) produce lipase and lecithinase and 5 isolates (61%) produced protease and esterase.

Keyword: ***Listeria moncytogenes,* PCR identification**

عزل وتشخيص بكتريا(*Listeria monocytogens*).بتقنيةPCR من بعض مصادر الاغذية في مدينة اربيل

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الخلاصة :

اجريت الدراسة الحالية لتقييم بعض الأطعمة في الأسواق المحلية لمركز مدينة أربيل و مدينة كويه للفتره من أكتوبر/تشرين الأول 2010 إلى مارس/آذار 2011. جمعت 225 عينة من نماذج غذائيه مختلفة بشكل عشوائي من الاسواق المحلية. تَضمّنتْ العيناتُ المدروسةُ 70 عيّنةِ من لحمِ الدجاجِ الخامِ، 55 عيّنةِ من الحليبِ الخامِ، 50 عيّنةِ من الجبنِ و50ْ عيّنةِ من اللحومِ الحمراءِ الخامِ. فُحِصتْ جميع العينات في الدراسة الحالية لمدى تلوثها بجرثومة اللستيريا وخصوصا(*Listeria monocytogens*). طبقاً لإختبارات حركة جرثومة *L. monocytogens* و انتاجها لانزيم الحال للدم وقدرتها على تخمير السكريات الى جانب استخدام تقنية تفاعل البلمرة المتسلسل (PCR)

ISSN 2072-3875

للحصول على تضخيم الحمض النووي عشوائيا باستعمال المبائ الجينية الخاصة للجرثومة في عملية العزل المختبري، تم تشخيص 8 عزلات للجرثومة، بمعدل عزلة واحده وبنسبة 2% من عينات الجبن، وعزلتين وبنسبة 4% من عينات مِنْ اللحمِ الأحمرِ، وخمسة عزلات بنسبة 7.3 % من لحم الدجاج. تراوحت نسب التلوث لعينات لحمِ الدجاجِ، حليب خام، جبن ولحم أحمر بجرثومة (Listeria) 20، 9، 20 و22 % على التوالي. على اساس قدرة الجرثومة المعزولة (*Listeria monocytogens*) لإنْتاج بَعْض الإنزيماتِ ذات الفعالية في امراضية البكتيريا مثل انزيم الكاتاليز(Catalase)، والانزيم الحال هابوريت(Hippurate)، والانزيم الحال للدم (hemolysin)، والإنزيم الحال للبروتينات(Protenase) ، والانزيم الحال للسيثين (lecithinase)، والانزيم الحال للحمض النووي(DNase) والانزيم الحال للدهون(lipase) والانزيم الحال للاسترات الدهني(esterase). فقد اظهرت النتائج ان جميع عزلات المشخصة الثمانية 8 كانت منتجة لانزيمي الكتاليز والحال للهايبوريت (100 %) ، و6 عزلات فقط (75%) كانت لانزيمي DNase وHemolysin ، و7 عزلات (87 %) منتجة لانزيمي lipase وlecithinase و5 عزلات (61 %) منتجَه لانزيمي Protease وesterase.

Introduction :

Listeria monocytogenes is a Gram positive, facultatively anaerobic, motile, nonsporeforming rod, which is capable of causing serious invasive illness (listeriosis) in both humans and animals (Roberts and Greenwood, 2003). The organism grows over a wide temperature range from 1 C to 45 C, with an optimum around 37 C. *L. monocytogenes* can grow at pH values between 4.4 and 9.4, and at water activities ≥0.92 with sodium chloride (NaCl) as the solute (Dimic *et. al*, 2010). *L. monocytogenes* is widely found in the environment and has been isolated from avariety of sources, including soil, vegetation, meat, silage, faecal material, sewage, and water(Dimic *et. al*, 2010). This bacterium is resistant to various environmental stresses, such as highly salty or acid solutions, which allows it to survive longer under stressful conditions than most other non-sporeforming bacteria of foodborne disease concern (Tolga *et, al*. 2010), and can survive for a long time in foods, processing plants, households, at refrigeration temperatures. Although it commonly exists in raw foods of both plant and animal origin, it is also present in cooked foods due to post-processing contamination, from food processing environments; especiallyt those that are cool and wet (Tompkin, 2002). There are seven species within the genus *Listeria* (*L. monocytogenes, L. ivanovii, L.innocua, L. seeligeri, L. welshimeri, L. grayii, and L. murrayi*), only two, *L.monocytogenes* and *L. ivanovii*, are pathogenic, the former causing disease in both humans and animals, (Roberts and Greenwood, 2003; Swamina, 2006). Almost 99% of human listeriosis has resulted from consumption of contaminated foods (Mead et al., 1999). Listeriosis accounts for less than 1% of cases of foodborne illness, but for around 28% of the deaths, (Kumar,2011). Listeriosis affects most often the pregnant women, fetus, elderly and immune compromised by affecting the central nervous system (CNS), and blood circulation (Swamina, 2006) . *Listeria monocytogenes* is able to form biofilms especially if the nutrient conditions are quite favorable (Adriao *et al*,2008).

The purpose of this study was to isolation and identification of Listeria monocytogenes from different kinds of local food in Erbil city .

ISSN 2072-3875

Material and Method

Collection of samples: Two hundred and twenty five (225) Samples of soft cheese , raw milk , red meat and chicken meat were collected from and around different market areas in Erbil City for a period of 6 months and examined for the presence of *L.monocytogenes* in some local food samples which were:-

(a) Raw milk (55 samples),(b) soft cheese (50 samples),(c)red meat(50samples),(d) chicken meat(70 samples) were collected under sterile conditions during 2010–2011.

Enrichment of Listeria Species: Listeria spp. were isolated according to two steps (primary and secondary) enrichment (Pagotto *et al.*, 2001) :

Primary enrichment: 25 gm or ml of food sample was added to 225ml of LEB1 then homogenized by homogenizer after that incubated the LEB culture at 30˚C . for 48 hrs .

Secondary Enrichment: After 48 hrs of the primary enrichment step, the LEBI culture mixed using vortex mixer. 10 ml of the culture was inculcated with 0.1 ml of the LEB1 culture, and incubated at 35˚ C for 24-26 hrs .

Isolation of Listeria Spscies: Listeria spp. were isolated by using the PALCAM (PAL) and Oxford (OXA)agar as selective culture media for Listeria isolates . A positive LEB II culture streaked onto PALCAM and Oxford agar , while a negative LEB II culture identified by showing the straw color of newly made broth was re incubate another 24hrs , a positive MFB streaked on two different plating selective media .Inoculated (OXA) and (PAL) streaked agar incubated at 35˚C for 24-48 hrs (Warburton *et al.* , 2003).

Purification of Listeria Isolates:*Listeria* suspected colony was streak on PALCAM agar without addition of antimicrobial agents and according to cultural characteristics of the coloy the confirmation test was done. The identification of *Listeria* specie was based on colors, sizes and colonial morphology (Macroscopic) patterns.

Biochemical Tests and Enzymes Detection

Catalase Test:A drop of hydrogen peroxide (H_2O_2) 3% concentration was placed on a clean dry slide. Then a fresh colony of the growth Listeria on nutrient agar medium was placed on the drop of hydrogen peroxide, the positive result indicated by of air bubbles as a result of O_2 production (Chukwu, 2007).

Oxidase Test: A fresh purified colony was streak on a piece of filter paper, and a drop of 1% colorless tetramethyl-p-phenylelediamine dihydrochrolide (TPD) reagent was added . A positive result was indicated by appearance of the purple within 10-30 seconds(Collins and Lyne ,1987).

Heamolysis on Sheep Blood Agar: Purified fresh colony were streak on (5-7%) sheep blood agar, then the media incubated for 48 h at 35˚C ,the positive result indicated by appearing of narrow zone of β-hemolysis around the colony (ChuKwu, 2007).

Carbohydrates Fermentation Test: All suspected *Listeria*- like organisms were tested for their ability to ferment Glucose, Rhamnose, Xylose, Lactose, Sucrose, melibiose, galatose, salicin and Mannitol. The suspected *Listeria* colony was inculcated to phenol

ISSN 2072-3875

-red -peptone broth containing 1% percent of used sugars for their fermentation tests and incubated at 37ºC for 24 hr, the positive results indicated by changing of color indicator from red to the yellow (Harley-Prescot , 2002).

Confirmation of *L. monocytogenes* by PCR

Genomic DNA Extraction

Freshly grown bacteria (approximately 10^8 cells/ml) were boiled in 1x Tris-EDTA buffer for 10 min followed by centrifugation at 2,000 rpm for 10 min. to remove denatured proteins and bacterial membranes. The presence of genomic DNA in all prepared samples was confirmed by 0.7% agarose gel electrophoresis followed by staining with ethidium bromide (**Dmitriy** *et al.*, 2006). *Listeria monocytogenes* isolates were detected by PCR assay using primer sequence mentioned in table (1).The samples were repeatedly propagated in the Fraser broth, cultured on selective media PALCAM and Oxford (Oxoid,Hampshire, U.K.). Suspect colonies of *Listeria* spp. were confirmed using **PCR identification of *L. monocytogenes***, 2 primers were selected based on the invasive association protein gene (iap) for *L. monocytogenes* as show in table (1).

Table(1) Primer sequence for detection of *L. monocytogenes*.

Species	Primer sequence (3' to 5')	Size	Reference
L. monocytogenes	**Forward TTA TAC GCG ACC GAA GCC AAC**	**660bp**	**Bubert *et al.*, 1999**
	Reverse CAA ACT GCT AAC ACA GCT ACT A		

The primer was supplied by Cinn Gen Company in lyophilized form. Cinn Gen company protocol was adopted for the primer resuspension by bringing the final concentration (10µM) of the primer with DNase free deionized distilled water. All PCR reactions were performed in a final volume of 25 µl using 2 µl of extracted DNA as template and distilled water was added to one PCR tube as negative control. Each reaction mixture contained 12.5 µl Master Mix and 1 µl(10µM) of forward primer ; 1µl(10µM) of reverse primer and 8 µl of Ultra-Pure DNase/RNase-Free distilled water. The DNA amplification reactions were performed in thermal cycler. The cycling conditions for PCR illustrated in table (2).

Table(2) :Thermal cycling protocols for detection of *L. monocytogenes*.

Species	InitialDenaturation	Denaturation	Annealing	Extension	ExtensionFinal
L. monocytogenes	**95˚C, 5 minutes**	**95˚C 45 seconds**	**50˚C 1 minute**	**72˚C, 1 minute**	**72˚C 10 minutes**
		Repeated for 31 cycles			

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Detection of PCR products (Gel Electrophoresis)

DNA samples were monitored using horizontal agarose gel electrophoresis according to Bartlett,(2001)

Results and Discussion

Pre-enrichment for samples in half fraser broth (primary listeria enrichment broth LEB1) which contain the selective agents acriflavin HCL, used to inhibit gram-positive cocci, nalidixic acid which inhibits Gram- negative bacteria and cycloheximide as antifungal. This step helps the recovery of stressed *Listeria* cell in the food samples (Kumar, 2011), secondary listeria enrichment broth LEB11 , in this broth the concentration of selective agents are double. Positive fraser broth has darkened and may be black, dark brown or dark green .a negative MFB has the straw color of newly made broth as shown in fig (1),these broth contain selective supplements which inhibit unwanted bacteria in the food samples which usually contain other bacteria as well. On the agar PALCAM (PLA) plates, *Listeria* colonies appeared grey–green. The colonies had black–sunken centers with a black halo against a cherry red background. PALCAM Listeria Selective Agar is recommended for the isolation of *Listeria monocytogenes* from foods, while inhibiting Gram-negative and most of the Gram-positive accompanying bacteria. The selectivity of the medium results from its content of polymyxin, acriflavin, ceftacidim, and lithium chloride. *Listeria monocytogenes* breaks down the esculin in the medium to glucose and esculetin, which forms an olive-green to black complex with ferric ions which stains the colonies of *Listeria monocytogenes.* (Walter, 2000). On Oxford agar *Listeria* showed the colonies grey–green. The colonies had black–sunken centres with that were surrounded by black halos. Selectivity is provided by the presence of lithium chloride in the formula also it is increased by adding various antimicrobial agents to the base. Incorporating these agents into Oxford Medium Base will completely inhibit gram-negative organisms and most gram-positive organisms at 24 hrs. incubation (Bille *et al* .,1999; Chukwu ,2007)

Fig (1) :L. spp. on Oxford agar

Fig(2)L. spp on PALCAM agar

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Confirmation Tests for identification of Listeria species Isolates

Listeria species at16- 24hrs.old, cells appear gram-positive reaction like rods, arranged singly, in short chains, in pairs at V-form angles and in groups that were parallel to each other. The organism could be rod- shaped, coccoi or filamentous. And this shape depends on nutrients, environmental and cultural conditions (Hass and Kreft, 1988).

Formation of bubbles is indicated a positive reaction of listeria species to catalase **(**Chukwu,2007**).** *listeria* species give a typical umbrella growth pattern on the motility test medium which is semisolid medium near the microaerophllic subsurface of the medium at (25C˚) as show in fig.(3). The bacteria exhibits a characteristic tumbling motility using peritrichous flagella at 20˚-25˚C and it is not motile at 37˚C , the gene that encoded flagellin are best expressed at 2°C to 25°C and *down* regulated at 37°C (Peiris, 2005;Brugere-Picoux, 2008)

Fig (3): Umbrella motility for Listeria spp.

Biochemical examination of *listeria monocytogenes*

All isolated *L species* strains subjected to routine laboratory biochemical tests for identification and to show their biochemical characteristics, the identification were confirmed by sugar fermentation.

The result showed that *L.monocytogenes* fermented salcin ,lactose ,galactose and rhamnose, and not fermented manitol, xylose,sucrose , and melbiose. All isolated strains hydrolyzed asculin to ascultin ,which reacts with ferric ammonium citrate producing a black precipitate coloring the media and this result agrees with (Fraser &Sperber ,1988). All isolated *L. monocytogenes* were positive for catalase and negative for oxidase,these are rapid for primary differentiation of listeria from other bacteria and important in pathogenicity of the bacteria due to remove H_2O_2 in macrophage (Low & Donchie ,1997). As shown in table (1) (75%) of isolated Listeria monocytogenes were produce hemolysin which is one of major virulence determinant of L. monocytogenes ,it is member of pore forming cholesterol-dependent cytolysin toxin family (Gedde *et al* .,2000)

The result show in table (3) that (61%) of *L.monocytogenes* were hydrolyzed tween 80 and converted it to oleic acid by esterase enzymes and this activity was indicated by

ISSN 2072-3875

producing clear zone around the colony (Dimic, *et. al.2010*). (61%) of *L.monocytogenes* strains were hydrolyzed casein, this result agree with Marquis, et.al. (1995) who reported that (58%) of *L. monocytogenes* were positive for protease.on the other hand (84%) of *L.monocytogenes* were positive for lecithin production.Vazquez *et al*., (1992) who reported (81%) of L. monocytogenes were positive for lecithinase and demonstrate the role of enzyme in cell to cell spread and in virulence of the organism.75% of the strains were positive for DNase this result resemble those of Al-Taey(2004) .

Table (3): biochemical characteristic of *L.monocytogenes*

Biochemical tests	No. of positive isolates(%)
Catalase	**8(100%)**
Oxidase	**0**
Hemolysin production	**6(75%)**
Lipase	**7(87%)**
DNase	**6(75%)**
Protease	**5(62%)**
Esterase	**5(61%)**
Hippurate hydrolysis	**8(100%)**
Esculin hydrolysis	**8(100%)**

Identification of *L. monocytogenes* isolates by Polymerase Chain Reaction (PCR)

Eight isolates *L. monocytogenes* were identified by biochemical tests subjected to PCR, and all these isolates were successfully amplified the desired amplicon of 660 bp as shown in fig (4). The PCR was performed using primer pair of *iap* gene, the nucleotide sequences of invasive associated protein (iap) genes deduced from amino acid sequence of P60 proteins was used.

- A major 60-kDa extra-cellular protein 60, encoded by *iap* gene plays a vital role in intestinal invasion and *in vivo* survival and all the isolates of *L. monocytogenes* secrete a protein of 60 kDa as a major extracellular product (Kuhn and Goebel,1989) encoded by the *iap* gene .there are many studies used iap gene as primer , Zeng *et al.* (2006) used *iap* gene as PCR - target for the species specific detection of *L. monocytogenes* from various samples and Nayak *et al* .,(2010) used primer targeting *iap* gene was used for species specific detection *L. monocytogenes* isolates up to $2x10^1$ CFU/ml.

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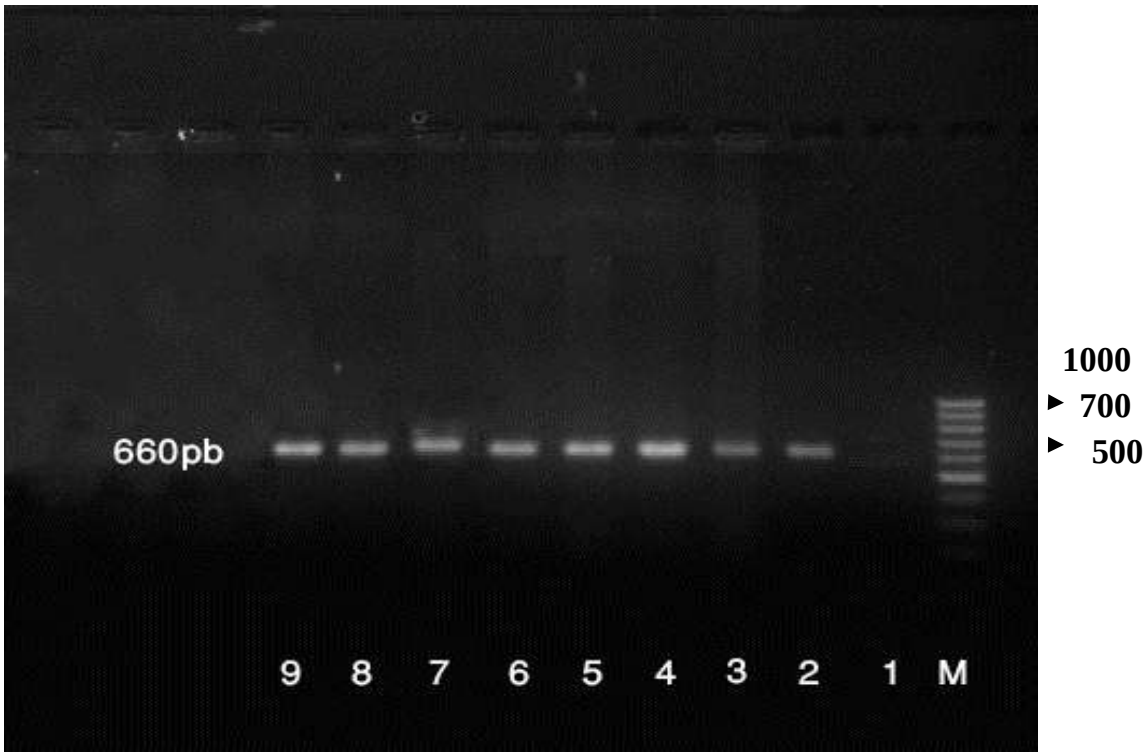

Fig.(4): Garose gel electrophoresis of PCR amplified iap 660 bp sequence from *L.monocytogenes* isolates . Lane M:1000 bp DNA ladder; Lane 1negative control; lane 2-9 samples positive for L.monocytogenes with iap(660 bp).

The presence of listeria species in food samples

Bacteriological investigation were done on (225) samples of (raw poultry meat ,raw milk, cheese and raw red meat).Eight isolates of *L.monocytogenes* were isolated from poutry meat ,red meat and cheese samples .The incidence of *Listeria* spp. was (16.58), (39) isolates of different species of *Listeria* (L.monocytogenes, L.innocua, L.grayi, L.welshmeri, L.ivanovii, L.seeligeri),with a percentage of 3.4%,7.23%,3.4%,0.85%,0.85% and0.85% respectively .as shown in fig (5,6).

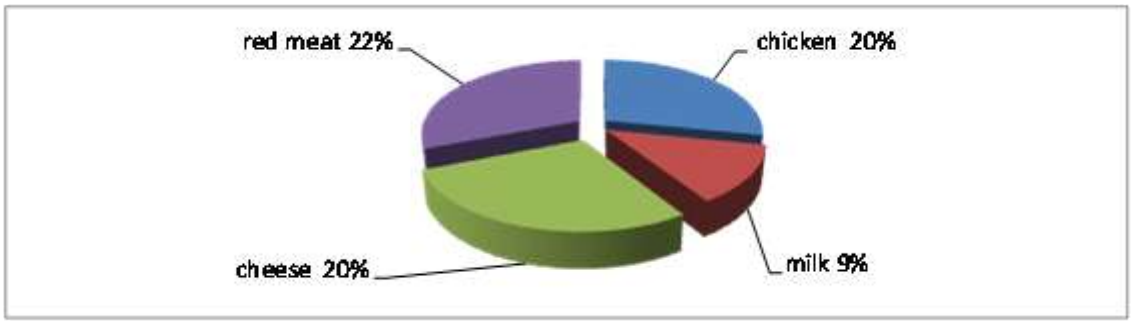

Fig (5): Pie chart showing the percentage of contamination of (225) food samples with *Listeria spp*.

ISSN 2072-3875

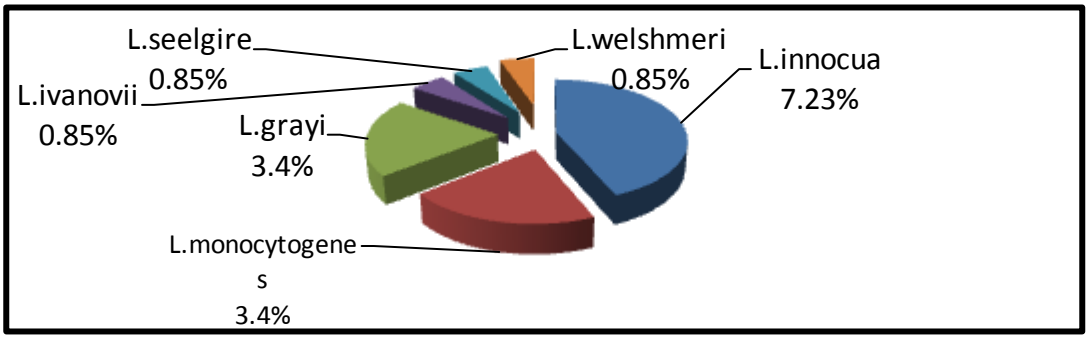

Fig (6):pie chart showing incidence of *Listeria spp*. in (225)samples of chicken meat,rawmilk,redmeatand and cheese.

Sammarco *et al.*, (2004) in Italy reported that *L monocytogenes* was not isolated from a total of (40) raw milk samples.

The incidence of *L .monocytogenes* was reported 5% in 80 raw milk samples from Ankara (Aygun and Pehlivanlar ,2006).

The higher prevalence of *Listeria* spp. (57.79-60.40%) and *L. monocytogenes* (49.25%) were reported by Jami *et al.*, (2010) reported the contamination of raw milk with *L. monocytogenes* was determined to be 4% of bulk tank milk in Iran.The occurrence of *listeria species* in (50) samples of different kinds of local cheese was 10 (20%) and *listeria monocytogenes* was1 (2%) as shown in table(5),also *listeria innocua* was isolate from (5) samples, L.grayi isolated from (2)samples, *L.welshimeri* isolated from (1)sample, *L.ivanovii* isolated from (1)sample and *L.monocytogenes* was isoltated from (1)sample.The incidence of *L. monocytogenes* in soft and semi-soft cheese varied from 0.50% to 46.00% (Pintado *et al.*, 2005; Colak *et al.*, 2007;Tolga *et al*, 2010; Jakobsen et. al.,2011) .

L. monocytogenes has been shown to adhere to several different food contact materials such as stainless steel, polypropylene and glass and the adhered cells show increased resistance to cleaning agents, disinfectants and heat ,all of which are used in the sanitation of the food processing plants , therefore the contamination of cheese during processing from environment is possible . The prevalence of the organism in cheese is hazardous to consumers because cheese is a ready to eat (RTE). From a total of (70) of raw Chichen (fresh and frozen) samples 19(27 .1%) were positive for *listeria* spp. Subdividing as5 (7.1*%) L.monocytogenes* , 5(7.1%), *L.innocua* , *L. grayi* 2(2.8*%), L.welshimeri* 1(1.4%), ,1(1.4*%)L.seeligeri* . This result is agreement with Ennaji *et al.*, (2008) in Morocco found that (20.3%) of poultry meat were positive for listeria spp., Among the strains of *L. species* isolates, (1.3%) were *L. monocytogenes,* (16.2%) *L. innocua* , and *L. welshimeri* (2.7%) were identified. Abd El-Malek et al (2010) in Assuit (Egypt) foud that listeria spp. was indicated in (52%) and (8%) *L.monocytogenes* in chicken legs and (54%) and (0%) *L.monocytogenes* in chicken fillet samples.The

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presence of *Listeria spp*. in (50) samples of red meat was 11(22%) , subdivided as 5(10%) *L. innocua* , 2(4%) *L.monocytogenes*, 2(4%) L.grayi , 1 (2%) *L.welshmire* , and 1(2%) *L.seelgeri* as shown in table (3) .

The incidence rate of Listeria spp. in red meat was (32%) isolated from of examined samples. *L. monocytogenes* occurred in (4%) and *L. innocua* in (28%) of tested samples in Issuit city in Egypt (Abd El-Malek et al .,2010), Nayak *et al ., (2010)* found that 10 (6.7%) samples were found positive for *Listeria* species, of which four (2.7%) were positive for *L monocytogenes,* two (1.3%) for *L. innocua,* three (2.0%) for *L. seeligeri* and one (0.7%) for *L. welshimer,* Contamination of the meat with *L. monocyto*results generally occurs after the slaughter and may come from the skin of the animals, the hands of the workers, the equipment and the tools used (Marinsek and Grebenc, 2002).

Prevalence of *L. monocytogenes* in food samples

In this study the pathogenic microorganism *L. monocytogenes* was isolated from 15 samples of raw milk and one sample of pasteurized milk. It was not found in any one of the collected samples of semi-finished and final products of milk (Table 2). The sourceof *L. monocytogenes* in raw milk is mostly the gastrointestinal tract of animals and the environment, skin of the teats, in particular (Graves *et, al.,*2009). Shedding of *Listeria* into milk due to chronic mastitis is less frequent.There are different opinions on the survival pasteurization treatment. At present, however, it is presumed that *L. monocytogenes* is killed by heating to 72°C at least 15 seconds. It is, nevertheless, true that so-called pasteurized milk was responsible for several pidemics of listeriosis (Jay, 1996).

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