Occurrence of *Listeria monocytogens* in raw milk of ruminants in Basrah province

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Abstract

The present study was performed on three hundred raw milk samples, 100 each from cows, sheep and buffaloes were collected from different places of Basrah city. 7.3 % of the samples were found to be positive for *Listeria* spp. Cow's milk was found to be more infected than other animals milk with this bacteria. All bacterial isolates were confirmed as *L. monocytogens* by colony characteristics, beta haemolysis, cold enrichment procedure, selective media, Anton test, tumbling and inverted pine tree motility and sugar fermentation tests. Most isolates were found to be sensitive to cefotaxine, sulfamethoxazol, chloramphenical and tobramycin. rifampicin was found to have less effect on these isolates. Effects of pH and temperature on bacterial growth were also studied to test the ability of this microorganism to survive in milk under severe conditions. The pH range for growth was from 4 to 9.5. The temperature range was between 4 – 45 ºC.

Keywords: Listeria, milk, cow, sheep, buffaloes.
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دراسة تواجد جراثيم اللستريا مونوسيتوجينز في الحليب الخام للمجترات في محافظة البصرة

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تضمنت الدراسة جمع ٣٠٠ عينة من الحليب الخام. جمعت ١٠٠ عينة من حليب كل من الأبقار والأغنام والجاموس ومن مناطق مختلفة من محافظة البصرة. وجد أن نسبة ٧,٣ % من العينات كانت موجبة لوجود جراثيم اللستريا كما وجد إن حليب الأبقار كان الأكثر إصابة من حليب بقية الحيوانات بهذه الجراثيم. تم تشخيص العزلات الجرثومية على إنها جراثيم اللستريا مونوسيتوجينز بواسطة الصفات المظهرية للمستعمرة وتحلل الدم وطريقة الاغناء المبرد والأوساط الزرعية الانتقائية واختبار انتون والحركة المشابهة لشجرة الصنوبر المقلوبة واختبار تخمر السكريات. وقد درست حساسية العزلات لعدد من المضادات الحياتية ووجد انها حساسه تجاه ال cefotaxine, sulfamethoxazol, chloramphenical, tobramycin وكان المضاد الاقل تاثيرا على العزلات هو rifampicin كما درس تأثير الأس الهيدروجيني والحرارة على نمو الجراثيم لمعرفة قابليتها على النمو في الحليب تحت الظروف غير المحببة ووجد ان الاس الهيدروجيني الذي تتحمله يتراوح بين ٤ – ٩,٥ والحرارة تراوحت بين ٤- ٤٥ درجة مئوية.

Introduction

Listeriosis is a food- borne disease that has become a major concern for the food industry and public health authorities in developed countries (1,2). Several outbreaks of listeriosis associated with the consumption of milk and dairy products have occurred since 1980 and are causing great concern to the dairy industry, owing to the number of cases and the nearly 30% over all mortality rate of these outbreaks (3). *Listeria monocytogenes* may directly contaminate milk as a consequence of listerial mastitis, encephalitis, or *L.monocytogenes* related abortion in cattle, and asymptomatic cows can also shed it in their milk for many months (4,5). It became appearent that the disease also affects humans, and rise during the 1980s. There was rising in the numbers of human cases in several countries (including the UK) together with evidence for food borne transmission of this disease has been much renewed interest

in this subject (6). The disease Listeriosis most often affects the pregnant uterus, the central nervous system, or the blood stream. In non-pregnant humans, Listeriosis usually presents as meningitis or septicemia in the Immunocompromised or elderly. During pregnancy, infection can spread to the foetus and may precipitate a stillbirth or the birth of a severity ill infant. The present study was done to investigate the occurrence of *Listeria monocytogenes* in cows, sheep and buffaloes milk in Basrah province and to study the sensitivity of *L. monocytogenes* to some common used antibiotics.

Materials and methods

Collection of samples

A total of (300) raw milk samples of (cows, sheep, and buffaloes were collected from farmers houses of different places at Basrah city during August 2006 to June 2007.

All samples of milk were collected according to (7). Milk collected randomly in sterilized condition by washing the udder by diluted saffelon solution. The area, then sucked up by clean cotton and the milk collected in sterile screw caped bottle after leaving the first drops. Milk were transmitted to the laboratory by cooling box. Samples were examined for the presence of *L. monocytogenes* immediately without further storage.

Isolation of bacteria from milk samples

Direct procedure: 0.1 ml of milk samples were streaked on surface of selective agar plates containing tryptose agar medium supplemented with 40µg /ml nalidixic acid., 30U /ml polymixin B sulphate, and the plates were incubated at 35ºC for 48 hrs (8).

Enrichment procedure: This method was done similarly to that described previously (9). 10 ml of milk was added to 90 ml of enrichment broth (EB) Tryptose broth containing above antibiotics and incubated at 35^{o}C for 24 hrs. In addition, 1.0 ml of EB culture was diluted with 9.0 ml of 0.5% KOH, mixed briefly, and streaked on selective agar plates and another selective plates containing tryptose agar + 40 µg/ml nalidixic acid + lithium chloride 0.5 gm /L (10).

Cold enrichment procedures: Ten ml of each milk sample were inoculated into 90 ml 0f Tryptose Broth (TB) which was kept at 4 ºC for 4 weeks (11). At the end of 7, 14, 21 and 28 days, 0.1 ml of this culture was plated on two types of selective agar plates mentioned previously. In addition, at the end of each week, 1ml of TB culture was inoculated into 9 ml of EB, containing (potassium thiocyanate 3.75% + Nalidixic acid 100 mg /ml) and incubated for 24 hrs at 35 ºC. 0.1 ml of the last culture was inoculated on both types of selective plates.

Identification of *Listeria monocytogenes*

Representative colonies of this bacteria were examined microscopically by the Gram staining technique and Henry technique, using obliquely transmitted microscope (12). Motility test and tumbling motility characteristic of *L monocytogenes* (13,14), blood haemolysis (15) and CAMP test with *Staphylococcus aureus* were investigated.

Biochemical testes: All biochemical tests were done according to (16,17).

Growth at various incubation temperatures and pH value: Pure culture of *Listeria monocytogenes* was streaked on nutrient broth (supplemented with 0.2% glucose). Cultures were incubated at different incubation temperatures (viz-4ºC, 25ºC, 37ºC, 45ºC and 50ºC). The plates were incubated for 3-7 days and examined for bacterial growth (13). For pH tolerance, bacterial strains were streaked on Tryptose agar medium having different pH value (3,4,5,5.5,6.5,7.5,8.5,9.5 and 10). Plates were incubated at 37ºC for 24- 48 hrs, and the growth were observed daily. pH were adjusted using NaOH (1N) and HCl (1N).

Anton's eye test: A small part of *listeria* colony was suspended in 1ml sterile saline. A drop was instilled into the conjunctiva of a rabbit. Inflammation and swelling of eyelids with a discharge were observed within 6 days. This test is available aid to determine the pathogencity of *L.monocytogenes* (14).

Antimicrobial susceptibility test: Antibiotics usceptibility test was done using antibiotic disc diffusion method (18).

Results

Total of (300) samples of raw milk were collected (100) from cows, (100) from sheep and (100) from buffaloes. The high incidence of *Listeria* was found in cow milk samples (11%) followed by sheep milk (8%) and buffaloes milk (3%) as shown in (Table 1). All colonies of *L. monocytogenes* isolated from raw milk showed typical 1-2 mm diameter and appeared cream-gray on blood agar. In addition, all isolates of *L. monocytogenes* produced the characteristic tumbling motility (inverted pine tree) when it was growing in brain heart Infusion broth at room temperature. All isolates of *L. monocytogenes* produced haemolytic reaction.

The distribution of *Listeria* isolates According to periods of collection

The distribution of Listeria according to the months of the study is shown in Table 2. In this table the highest rate of *Listeria* isolation was observed in winter months (Dec.,

Jan., Feb), and it reached 10.8% and the lowest rate (3%) was found in Sep, Oct and Nov. periods.

Table 1: Percentage of *L. monocytogenes* recovered from raw milk samples under study.

Samples Source	No. of examined samples	Positive samples	
		No	%
Cows milk	100	11	11
Sheep's milk	100	8	8
Buffaloes milk	100	3	3
Total	300	22	7.1

X2=4.807, P> 0.05.

Table 2: Percentage of *L. monocytogenes* from raw milk according to periods of collection.

Month of collection	No. of samples	No of isolates	%
Sep., Oct., Nov./2006	100	(3)	3
Dec., Jan., Feb./2006-2007	120	(13)	10.8
Mar., Apr., May /2007	80	(6)	7.5

X2=4.227, P> 0.05.

The distribution of Listeria isolates According to regions

A total of 300 samples were collected from six regions of Basrah city. The highest level of incidence of Listeria isolate (10%) was found in region 4 followed by region 1. The lowest one (2.5%) was found in region 3 as shown in (Table 3).

The distribution of *Listeria* isolates according to method of isolation and the types of selective medium

All milk samples were tested by three types of methods of isolation; the direct methods, the enrichment method and the cold enrichment method as shown in (Table 4). The highest numbers of isolates were isolated by cold enrichment procedure (54.2%) followed by enrichment procedure (36.8%), and the lowest one is the direct procedure (9.2%).

Selective media also found to influence the number of *Listeria* isolates as shown in (Table 4). Using Lithium chloride in addition to nalidixic acid in selective media have greatly affect on other bacterial strain and led to isolation percentage 50% of isolates in comparison to nalidixic and with polymixin B.

Table 3: Percentage of *L. Monocytogenes* from raw milk According to Regions of collection.

Region	No. of samples	No. of isolates	%
1. Qurna (Rular and marshs)	65	6	9.23
2. Garamt- Ali (Rular and marshs)	75	7	9.33
3. Al-Zubair (Desert)	40	1	2.5
4. Hi Al- Hussain (Urban)	40	4	10
5. Shatt Al- Arab (Rular)	40	2	5
6. Abu Al-Khasseb (Rular)	40	2	5
Total	300	22	

X2=3.22, P> 0.05.

Identification *Listeria* Species

All 22 *Listeria* isolates were subjected to several tests for species identification**.** (17). All isolates were identified as *L. monocytogenes* as shown in (Table 5).

Biochemical tests: All bacterial isolates were subjected to different biochemical tests. All these isolates showed similar results and having, positive results for gram stain, citrate, TSI, catatase and fermentation of glucose, manitole and rhaminose; other tests like oxidase, indole test, urea test, gelatin test and sugar fermentation were found to be negative.

The effect of pH and temperature on growth of *L. monocytogenes*

The effect of pH value and temperature on growth of the *L. monocytogenes* was studied (Table 6). The pH range for growth was found from 4 to 9.5. The temperature range between 4 – 45 ºC.

Table 4: Percentage of isolation of *L. monocytogenes* according to method of isolation and type of selective media.

Method of isolation	No.	%	Type of selective medium	No.	%
Direct method	2	8.26	Tryptose agar + nalidixic acid + polymixin B	4	18.1
Enrichment method	8	36.8	Tryptose agar + nalidixic acid + lithium chloride	11	50
Cold enrichment method	12	54.2	Tryptose agar + nalidixic acid + pot.thiocyanate	7	31.8
Total isolates	22			22	

X2=12.123, P< 0.01.

Antibiotic susceptibility tests

Fifteen different antibiotic discs were used for indicating the susceptibility of *L. monocytogenes* to this antibiotic. Most isolates were found to be sensitive to cefotaxine, sulfamethoxazol, chloramphenical and tobramycin. rifampicin found to have less effect on these isolates (Table 7).

Table 5: Identification of *L. monocytogenes.*

Strain No	Haemolysis	Production of acid		CAMP
		Rhamnose	Xylose	*S. aureus*
All isolates (1-22)	+	+	-	+

X2=5.045, P> 0.05, (+ refers to positive result, - refers to negative result).

Table 6: Effect of pH and temperature on level of growth of *L. monocytogenes*.

Temperature C	Growth	pH value	Growth
4	++	3	-
20	++	4	+
37	++	5	++
45	++	5.5	++
50	_	6.5	++
		7.5	++
		8.5	++
		9.5	++
		10	-

(- no growth, +weak growth, ++ good growth).

Table 7: Susceptibility and resistance of *Listeria monocytogenes* to some antibiotics under study.

Antibiotic	Source of listeria monocytogenes							
	Cows milk 11		Sheep milk 8		Buffaloes milk 3		Total	
	S* (%)	R* (%)	S (%)	R (%)	S (%)	R (%)	S (%)	R (%)
Vancomycin Vanc30	6 (54.54)	5 (45.5)	8 (100)	0 (0)	3 (100)	0 (0)	17 (77.27)	5 (22.72)
Lincomycin Linc 2	8 (72.72)	3 (27.27)	8 (100)	0 (0)	3 (100)	0 (0)	19 (68.36)	3 (13.63)
Cefotaxime Cef 30	11 (100)	0 (0)	7 (87.5)	1 (12.5)	2 (66.6)	1 (33.3)	20 (90.9)	2 (9.09)
Sulfamethoxazol Methox 25	11 (100)	0 (0)	7 (87.5)	1 (12.5)	3 (100)	0 (0)	21 (95.45)	1 (4.54)
Nitrafurantin Nitr 300	8 (72.72)	3 (27.27)	7 (87.5)	1 (12.5)	3 (100)	0 (0)	18 (81.81)	4 (18.18)
Rifampicin Rif 30	0 (0)	11 (100)	1 (12.5)	7 (87.5)	1 (33.3)	2 (66.6)	2 (9.09)	20 (90.9)
Chloramphenicol Chlor 50	10 (90.9)	1 (9.09)	8 (100)	0 (0)	3 (100)	0 (0)	21 (95.45)	1 (4.54)
Erythromycin Erth5	8 (72.8)	3 (27.2)	6 (75)	2. (25)	2 (66.6)	1 (33.3)	16 (72.72)	6 (27.27)
Cloxacillin Clox 5	7 (63.6)	4 (36.3)	8 (100)	0 (0)	2 (66.6)	1 (33.3)	17 (77.27)	5 (22.72)
Tobramycin Tob 10	11 (100)	0 (0)	7. (87.5)	1 (12.5)	3 (100)	0 (0)	21 (95.45)	1 (4.54)
	X2=45.833, P <0.01 H.S		X2=36.831, P< 0.01 H.S		X2=10.8, P> 0.05 N.S		X2=766.638, P< 0.01 H.S	

* Number of isolates, S: sensitive, R: resistance.

Discussion

The prevalence of the *L. monocytogenes* in cows' milk samples was (11%) this finding was high when compared with those recorded (1.25–7%) by other studies (8-10,19-22).

The presence of *L. monocytogenes* was found to be higher in cow milk samples in comparison to buffaloes milk samples. This may be due to the ability of buffaloes to resist to disease than cows or due to the nature and composition of milk.

These difference incidence may be due to differences in places or seasonal variation in milk samples collection or methods of isolation and adaptation of the bacteria to diverse ecological inches as well as the state of hygienic animals (19).

The recovery of *L. monocytogenes* from samples collected from Hi Al Huassian was found to be highest and the lowest recovery was found in Al- Zubair. This finding may be as a result of the dryed weather of Al- Zubair area, since hot weather and dryness could affect the bacterial cells.

The high prevalence of *L. monocytogenes* in cold weather months, and decreasing in hot weather months, may be related to feeding practices, herd arrangement, or to some unknown factors affecting either animal and bacteria relationship or bacteria and environmental relationship or both (10). This finding agreed with the results of other workers (23,24).

The use of enrichment and cold procedures gave more isolates than direct method. Most of the positive isolation by the enrichment and cold procedures were from the end

of 21st day incubation at 4°C. There was a considerable variability in the efficacy of two enrichment and cold procedures in the isolation of *L. monocytogenes*. In this study two enrichment and cold procedures were much more productive than direct method.

The addition of lithium chloride instead of polymixim B increases the recovery of *L. monocytogenes* from the samples. This indicates the wide effect of Lithium Chloride on microbial biota rather than *Listeria* (25).

The effect of some physical factors on growth of *Listeria* were studied. The ability of Listeria to grow at various range of pH (4-9.6) indicates that this bacteria which can grow in milk has different pH value (26).

Listeria can grow on different temperature. This phenomenon help to isolate this bacteria from mixed population since it can grow at low temperature. This also indicates the risk event of keeping milk at refrigerator temperature (13).

The results of antibiotic susceptibility tests of 22 *L. monocytogenes* indicates that these isolates were sensitive to most antibiotic agents. All isolates were sensitive to sulfamethoxazole. Chloromphincol and tobramycin. Resistance to rifampicin was presented in 90.9% of the isolates and to erythromycin in 27.27%. These results indicate that the animals (cows and sheep) which were sources of the *L. monocytogenes* isolates tested in this study, may have not received antibiotics previously. Many Antimicrobial drugs inhibit this bacterium *in vitro* such as ampicillin, erythromycin and sulfamethoxazole (18). On the other hand, isolates with multiple resistance to 2-9 antibiotics were also observed. It is known that resistant isolates have ability to survive longer period in environment than the sensitive isolates, and then can be transferred these resistance with high frequencies. Moreover, they may have a selective advantage in environment. Therefore, the occurrence of multiple antibiotic resistant among *L. monocytogenes* reported here would be are health threating because this organism is associated with a large number of foodborne outbreaks and the choice of a suitable antibiotic becomes more difficult.

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