Serology Unit, Animal Health Research Institute,
Dokki, Giza.

CONTAMINATION OF SOME LOCAL FISH WITH *LISTERIA MONOCYTOGENES* AND STUDYING ITS CHARACTERIZATION AND CONTROL

(With 7 Tables)

By

HALA S. IBRAHIM and HALA F. HASSAN*

*Dept. of Food Hygiene, Animal Health Research Institute, Dokki, Giza.

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مدى تلوث الاسماك المحلية بميكروب الليستيريا مونوسيتوجين ودراسة خصائصها والتحكم فيها

هالة سلطان ابراهيم ، هالة فريد حسن

تـم عـزل عشـرون عترة ليستريا مونوسيتوجين من بعض الاسماك المحلية من المحلات والسوبر ماركت فى خمس مدن مصرية وقد وجد أن نسبة العزل الكلية (٩,٣%) كانت أكثر نسبة عزل فى القرموط (٤,١٩%) والمكرونه (٢,٧٩%) والسردين (٢,٣٣%). وقد صنفت هـذه العـترات علـى حسب ثمانية خصائص ظاهرية منها تخمر السكريات ، وجد ان هذه العترات تختلف فى قدرتها على تخمر الدالسيت ولاكتوز ومالتوز ومانينول والنشا والزيلوز بينما لها القدرة على تخمر كل من الجلوكوز والسالسين وسكروز وتريهالوز والرامينوز ولها المقـدرة علـى تحمل التركيزات العالية من كلوريد الصوديوم من (٨-٢٠%) وقد وجد ان الاحمـاض العضوية ليس لها المقدرة على تثبيط نمو الليستيريا مونوسيتوجين خلال ٥-١٠ دقائق ولكنها لها المقدرة بعد ٢٤ ساعة برغم استخدام تركيزات مختلفة (٠,٥، ١، ٢و ٥%) ونسـبة مقدرتها على الاتحاد بصبغة الكونجو الحمراء وانتاج الليثيثين هى ٩٠ و٨٥% على الـتوالى. قد وضحت اختبارات الحساسية للمضادات الحيوية ان كل العترات المعزولة كانت مقاومـه للكولسـتين سلفات وانروفلوكساسين ونااليدكسك اسد واكرولينك اسد بنسبة ١٠٠% ولكـنها حساسـة ١٠٠% للكلورامفينيكول ثم يليه الاريثرومايسين (٨٠%) ، اموكسيسللين (٧٥%) ثـم اوكسىتتراسـيكلين (٧٠%) وسبروفلوكساسين (٦٥%). وقد وجد ان العترات المعـزولة احدثـت التهاب الملتحمة فى خنازير غينيا و١٦ عترة فقط احدثت نفوق الفئران خلال ٧ أيام وجميع العترات ادت الى تحلل كرات الدم الحمراء للحصان والخروف وخنازير غينيا والانسان ولكنها تختلف عند اضافة المانوز حيث كانت النسبة ٨٥، ٧٥، ٥٠، ٥٠ % علـى الـتوالى. قد وجد ان العد البكتيرى لميكروب الليستيريا المحقون فى السردين قد تاثر بالـتعرض لدرجـات الحرارة المختلفة فزيادة درجة الحرارة ووقت التعرض لها تؤثر على العـدد فعند درجة ١٠٠° م لمدة ٢٠دقيقة يؤدى الى القضاء عليها تماما اما عند ٧٠° م لمدة ٢٠ دقـيقه يقـل العدد فقط بينما عند ٥٠° م حتى مع زيادة الوقت لم يؤثر على العدد بينما

المعالجة بالاحماض العضوية اوضح ان زيادة تركيز الحمض يقلل معدل النمو وكانت علاقة تثبـيط النمو باستخدام الاحماض العضوية كالاتى (حمض الاكتيك ثم الاسيتك ثم الستريك) ومن الملاحظ فى هذا البحث ان عند استخدام تركيز ٠,٠٥% من الاحماض العضوية شجع نمـو الميكروب، وفى حالة استخدام انتى ميكروبيل ايس على السردين لم يقضى تماما على الميكروب بل قل عده من ١×١٠ ^٨ الى ١,٥ × ١٠ ^٥ و٢ × ١٠ ^٣ بعد ٦٠ و ١٢٠ دقيقة على التوالى.

SUMMARY

A total of 20 *L. monocytogenes* strains isolated from some local fish from different shops and markets located in 5 towns in Egypt. The total incidence of *L. monocytogenes* in raw fish was 9.3%, it was predominated in Clarias (4.19%) followed by Saurus (2.79%) then Sardines (2.33%). They were phenotypic characterized with respect to 8 characters, carbohydrate fermentation profiles showed variation in utilization of dalcite, lactose, maltose, mannitol, starch and xylose while glucose, salicin, sucrose, trehalose and rhaminose yielded positive in all strains. They were tolerant to NaCl at high salt concentrations ranged from 8% to 20%. The growth inhibition of *L. monocytogenes* in presence of organic acids not occurred within 5 and 10 minutes but occurred after 24 h incubation at 37°C even with different concentrations (0.5, 1, 2 and 5%). The positive percent of Congo red binding activity and Lecithinase production were 90% and 85% respectively. The drug susceptibility characterization of *L. monocytogenes* cleared that all isolates were resistant (100%) to colistin sulphate, enrofloxacin, nalidixic acid and oxolinic acid, but were susceptible to chloramphenicol (100%) followed by erythromycin (80%), amoxycillin (75%) then oxytetracycline (70%)and ciprofloxacin (65%). Pathogenicity in laboratory animals showed that 20 strains caused keratoconjunctivities in G. pig and only 16 strains killed the mice within 7 days. All strains were haemolytic to RBCs of horse, sheep, G. pig and human and only showed change in haemolysis with addition of D-mannose as follows 85, 75, 50 and 50% in horse, sheep, G. pig and human RBCs respectively. The count of *L. monocytogenes* inoculated in Sardine was affected by heat treatment where increasing time of exposure and degree of temperature reduced count as 100°C for 20 minutes completely eliminated *L. monocytogenes* , while at 70°C for 20 minutes only reduced count and having no effect at 50°C even with increasing time. Whereas treatment with organic acids revealed that when the acid concentration increased, the growth rate of *L. monocytogenes* decreased and the relative inhibition effect was

generally lactic > acetic > citric acid , form interest of this work that low concentration of organic acids (0.5%) enhanced growth of *L. monocytogenes*. Application of antimicrobial ice on Sardine not completely eliminated but reduced *L. monocytogenes* count from 1 X 10^8 to 1.5 X 10^5 and 2 X 10^3 after 60 and 120 minutes exposure respectively.

Key words: *Fish, Clarias spp., saurus spp., sardine, L. monocytogenes.*

INTRODUCTION

Fish is considered a very important diet for human due to its protein content and high level of calcium and phosphorus. Fish may be subjected to various forms of contamination by different microorganism including *L. monocytogenes* which is an opportunistic bacterial pathogen of man and animal. It has the ability to survive under extreme environmental conditions causing illness to animals and man called listeriosis, specially in pregnant woman, new born infants, children and adults whose immune system are weakened (Rocourt *et al.*, 2000).

More recently a new form of the disease has been recognized, it is characterized by mild disorders of the gastrointestinal system and short incubation period. *L. monocytogenes* has been involved in a food borne outbreaks of gastroenteritis (FAO report, 1999). Due to ubiquitous nature of *Listeria* spp. and its ability to grow in refrigerated temperature and low oxygen level, its isolation from fish is recorded by Kwiatek, (2004) who isolated it from raw fish.

The recent concern about decontamination of *L. monocytogenes* by physical and chemical methods that inhibit this bacteria to prevent future outbreaks as heat treatment or addition of certain substance that remove or minimize the *Listeria* load in fish and fish products included acetic and citric acid (Naser, 2003), antimicrobial ice containing chlorine dioxide (ClO_2) (Shin *et al.*, 2004) or addition of lactic acid alone or with hot water (Koutsoumanis *et al.*, 2004).

The investigation dealt with:

- The prevalence of *L. monocytogenes* in some local fish including Saurus, Sardines and Clarias Spp
- Studying some phenotypic characterization with respect to carbohydrate fermentation profiles, haemolytic activity, growth inhibition by sodium chloride, and organic acids (acetic, citric and lactic acid) Congo red binding activity, lecithinase production test, drug susceptibility and pathogenicity in laboratory animals (G. pig and mice).

- In addition to studying the effect of heat treatment, addition of acetic, citric and lactic acid and also antimicrobial ice on contaminated samples with *L. monocytogenes* was investigated.

MATERIALS and METHODS

Fish samples:

A total of 215 fish samples including 80 Saurus, 70 Clarias and 65 Sardines were purchased from different shops and markets of various size located in 5 towns in Egypt, collected in sterile polyethylene bags and transferred to the laboratory in an ice box then kept at 0- 4°C until tested within 24 h.

Bacterial strains:

Rhodococcus equi and *Staphylococcus aureus* for CAMP test were obtained from Animal Health Research Institute. *L. monocytogenes* microorganism was isolated during this study. The stock culture was maintained at -20°C in trypticase soy broth containing 10% glycerol.

Blood:

Blood was collected from horse, sheep, G. pig and human into tubes containing sodium citrate in distilled water per 9.0 ml of blood. Blood was diluted 1:4 with phosphate buffered saline (PBS) of pH 7.2 to test for haemagglutination (HA) and 1:4 with 1% D mannose in PBS test for mannose resistant haemagglutination MRHA.

Laboratory animals:

1- Mice:

A total of 100 albino white mice with average weight of about 18- 20 gm and aged 30- 35 days were used for detection of pathogenicity of *L. monocytogenes*.

2- G. pig:

A total of 20 G pigs were used for interaconjunctival infection for detection of conjunctivitis caused by *L. monocytogenes*.

Disinfectants :

- Acetic, citric and lactic acids, each was used in a concentration of 0.5, 1, 2 and 5%.
- Antimicrobial ice was obtained from some factories containing chlorine dioxide.

Isolation of *Listeria monocytogenes* from fish:

According to FAO (1992) 25 gms from each fish sample were added to 225 ml of Listeria enrichment broth (L.E.B.), then incubated at 30°C for 48 h. The enrichment culture was streaked on PALCAM selective agar and incubated at 30°C for 24- 48 h.

Identification and characterization of *L. monocytogenes* isolated from fish:

Colonies suspected to be *L. monocytogenes* were identified according to Koneman *et al.* (1996) and Quinn *et al.* (2002) and characterized according to Margolles *et al.* (2000) by Gram stain, tumbling motility, V.P, catalase, oxidase, haemolysis on horse blood agar and CAMP tests.

CAMP technique:

B haemolytic *Stayphlococcus aureus* and *Rhodococcus equi* cultures in parallel and diametrically opposite each other were streaked on sheep blood agar plate, where several test cultures were streaked parallel to one another, but at right angles to and between *S. aureus* and *R. equi* streaks. The plates were incubated at 35°C for 24- 48h then examined for haemolysis.

All *L. monocytogenes* give haemolytic reactions which are enhanced in the zone influenced by *S. aureus* streak.

characterization of *L. monocytogenes*

1. Carbohydrate fermentation profiles:

This test was performed by addition of single colony to 5 ml of basal liquid medium (meat extract 1%, sodium chloride 0.5% and bromocresol purple 0.02 g pH 6.8) containing 1% of the appropriate carbohydrate including glucose, dalcite, lactose, maltose mannitol, salicin, starch, sucrose trechalose, xylose, and rhamnase. Development of yellow colour after 24- 48 h of incubation at 37°C was considered a positive result.

2. Growth inhibition by sodium chloride:

Over night cultures on tryptic soya broth (T.S.B) were used to inoculate fresh T.S.B supplemented with NaCl in a range from 8 to 20% at 1% intervals then 200 µl of each inoculated media were dispensed into wells of microtitre plates and were incubated at 37°C for 48 h. To ensure the growth of *L. monocytogenes* a loopfull from each well was cultured on PALCAM agar as solid media and was incubated 30°C for 24h.

3. Growth inhibition by organic acids:

Over night cultures on T.S.B were inoculated to fresh T.S.B supplemented with one of each of the 3 organic acids (acetic, citric and lactic acid) at mentioned concentrations, then 200 µl of each suspension was dispensed into wells of microtitre plates that were incubated at 37°C for 48 h and ensured growth by cultivation on PALCAM.

4. Congo red (C.R) binding activity: Delgado-da-Silva *et al.* (2001).

Listeria monocytogenes strains were cultured into Congo red media, the reaction was best seen after 24 h of incubation at 37°C and then left at room temperature for additional 2 days. CR^+ was indicated by development of red colonies, CR^- colonies appeared white as did not bind to the dye.

5. Lecithinase production test: Delgado-da-Silva *et al.* (2001)

Listeria monocytogenes isolates were inoculated on the surface of egg yolk agar media and incubated at 37°C for 24 h. Positive result was indicated by appearance of colonies surrounded by zone of opacity.

6. Drug susceptibility testing: Finegold and Martin (1982) and Quinn *et al.* (2002).

On Muelleur Hinton medium the standard disk diffusion assay was used to determine susceptibility of the isolated *L. monocytogenes* to 12 antimicrobial agents (Oxoid), using the following discs, amikacin (30µg), amoxicillin (25µg), cefadroxil (30µg), chloramphenicol (30µg), ciprofloxacin (5µg), colistin sulphate (50µg), enrofloxacin (5µg), erythromycin (15µg), nalidixic acid (30µg), oxolinic acid (2µg) oxytetracycline (30µg) and streptomycin (10µg). The results were interpretated according to the manual supplied by the manufacture company.

7. Pathogenicity in laboratory animals:

All laboratory animals wereexamined bacteriologically and proved to be free from pathogens.

- Mouse lethality test: Stelma *et al.* (1987).

The mice were divided into groups (5 in each) according to number of isolates. Last group was kept as control and each mouse was inoculated with 0.1 ml of 10^{10} C F U of test strain of *L. monocytogenes* I/ P, each group was kept separately. All mice were kept under observation for 7 days. Number of deaths were recorded daily and reisolation of *L. monocytogenes* was done from dead mice. Strain that killed 3 or more mice was considered pathogenic.

- Anton test:

1 G. pig was used for each isolate. Instillation of G. pig eye with early pure culture causing conjunctivitis within 24- 48 h.

8. Haemolytic activity: Dominguez *et al.* (1986)

Two well developed colonies from sheep blood agar were suspended in 0.5 ml of saline solution Two fold dilutions were made in microtitre plate with U form wells containing 50µl of saline solution then 50 µl of bacterial suspension was added to the first well. 50µl from

well were transferred to another containing saline solution and so on. 100 µl of 3% of washed sheep RBCs were added to each bacterial dilution. Negative control well contained 100µ of washed sheep RBCs and 50µl of saline solution. The microtitre plates were incubated at 37°C for 16 h. The haemolytic activity was expressed as complete haemolysis unite C.H.U. (the inverse of highest dilution at which 100% haemolysis occur) and minimal haemolysis unite M.H.U. (the inverse of highest dilution at which haemolysis was detected). The test was repeated using washed G pig then human RBCs.

Preparation of *Listeria* inocula and fish inoculation: Koutsoumanis *et al.*,(2004).

A mixture of six *L. monocytogenes* strains showed various phenotypic characterization isolated from fish in this work, were grew on tryptic soya broth at 30°C for 24 h, then centrifuged at 2000 rpm for 15 minutes. The harvested cells were washed in 10 ml phosphate buffer saline, centrifuged as previously then the washed cells were diluted with P.B.S till obtain 10^8 CFU/ ml. Then 1 ml was inoculated to each fish sample (previously examined to ensure it is free from *L. monocytogenes*, and left to dry off for 15 minutes at 4°C. *Listeria* was counted using PALCAM agar.

Treatments used to control *L. monocytogenes*:

1- Heat treatment:

Fish were experimentally contaminated with *L. monocytogenes* and subjected to various degrees of temperature with different (increasing) durations in hot air oven that was considered as grilling in following temperatures 50, 70 and 100°C each with the following time 10, 15 and 20 minutes.

2- Addition of organic acids:

Each of acetic, citric and lactic acids was added in mentioned concentrations to fish contaminated with *L. monocytogenes* in polyethylene bags for 30 seconds, 1 and 5 minutes.

3- Application of antimicrobial ice:

Contaminated fish were put in plastic bag with antimicrobial ice and tested for time intervals of 60-120 minutes

RESULTS

Table 1: Occurrence of *L. monocytogenes* in some local raw fish.

Fish species	No. of examined fish	No. of + ve samples	% *	% **
Saurus	80	6	7.5	2.79
Clarias	70	9	12.86	4.19
Sardines	65	5	7.69	2.33
Total	215	20	28.05	9.3

* Percent was calculated according to the number of each species of the examined fish.
** Percent was calculated according to the total number of the examined fish (215).

Table 2: Characterization of *L. monocytogenes* isolated from local fish.

Test	No. of + ve isolates	No. of - ve isolates	% of positive
Carbohydrate profiles:			
Dalcite	13	7	65
Lactose	14	6	70
Maltose	15	5	75
Mannitol	10	10	50
Starch	16	4	80
Xylose	3	17	15
Growth inhibition by NaCl.	-	20	0
Growth inhibition by organic acids after: 5-10min : 24hr	- 20	20 -	0 100
Congo red binding activity.	18	2	90
Lecithinase production.	17	3	85
Drug susceptibility.			
Amikan 30	10	10	50
Amoxycillin 25	15	5	75
Cefadroxil 30	5	15	20
Chloramphenicol 30	20	0	100
Ciprofloxacin 5	13	7	65
Erythromycin 15	16	4	80
Oxytetracycline 30	14	6	70
Streptomycin 10	11	9	55
Pathogenicity test: a. keratoconjunctivitis in G. pig	20	0	100
b. Death of mice with 5 day	16	4	80

The percent was calculated according to the total number of strains (20).
Glucose, salicin, sucrose, trehalose and rhaminose were +ve in all isolates.
Sodium chloride was used in range from 8 to 20 % at 1% intervals for each strain in growth inhibition test.
Colistin sulphate, enrofloxacin, nalidixic acid, oxolinic acid were–ve in all strains.
Inoculation dose: 0.1 ml of 10^{10} CFU/mice I.P.

Table 3: Haemolytic characterization of *L. monocytogenes* isolated from local raw fish.

Source of RBCs	No. of HA+	%	No. of HA^+ with D-mannose	%
Horse	20	100	17	85
Sheep	20	100	15	75
G. pig	20	100	10	50
Human	20	100	10	50

The percent was calculated according to the total number of samples (20).

Table 4: Haemolytic titre of *L. monocytogenes* isolated form local raw fish.

Type of haemolysis	Complete haemolysis unit									Minimal haemolysis unit								
Titre	1/8			1/16			1/32			1/256			1/612			1/1224		
Species	S	G	H	S	G	H	S	G	H	S	G	H	S	G	H	S	G	H
+ve strai No.	0	16	16	10	4	4	10	0	0	0	15	17	10	5	3	10	0	0

S: sheep G: G pig H: human

Table 5: Efficacy of heat treatment on count of *L. monocytogenes* in Sardines.

Temp.	Count in relation to time		
	10 m	15 m	20m
50	3×10^8	3×10^8	3×10^8
70	3×10^8	1.5×10^6	4×10^6
100	1.5×10^5	3×10^3	0

Table 6: Efficacy of organic acids treatment on count of *L. monocytogenes* in Sardines.

Organic acids	Time	Concentration			
		0.5%	1%	2%	5%
Acetic acid	30 s	$3X\ 10^9$	$3\ X\ 10^7$	$1.5X\ 10^7$	$1.5X\ 10^5$
	1 m	$3X\ 10^9$	$1.5X\ 10^7$	$6X\ 10^5$	$1.5X\ 10^3$
	5 m	$1.5X\ 10^9$	$1.5X\ 10^3$	$3\ X\ 10^2$	$2X\ 10^1$
Citric acid	30 s	$6X\ 10^9$	$3\ X\ 10^8$	$1.5X\ 10^7$	$1.5X\ 10^6$
	1 m	$3X\ 10^9$	$1.5X\ 10^7$	$6X\ 10^6$	$1.5X\ 10^5$
	5 m	$1.5X\ 10^9$	$1.5X\ 10^5$	$3\ X\ 10^4$	$2X\ 10^3$
Lactic acid	30 s	$3X\ 10^9$	$3\ X\ 10^6$	$1.5X\ 10^4$	$1.5X\ 10^2$
	1 m	$3X\ 10^9$	$1.5X\ 10^4$	$6X\ 10^3$	$1.5X\ 10^1$
	5 m	$1.5X\ 10^9$	$1.5X\ 10^3$	$3\ X\ 10^2$	0

S = seconds m =minutes

Table 7: Efficacy of antimicrobial ice on count of *L. monocytogenes* in Sardines.

Time	Count
60 m	$1.5\ X\ 10^5$
120 m	$2\ X\ 10^3$

DISCUSSION

Listeria monocytogenes is of greatest concern from the public health point of view, consistently associated with human illness and linked to many outbreaks due to consumption of food. Dalton *et al.* (2004) in their studies about foodborne diseases outbreaks, found that the most frequently implicated vehicles in the 173 outbreaks were seafood and *L. monocytogenes* caused 40% of deaths.

The present study dealt with the occurrence of *L. monocytogenes* in some local fish including Saurus, Clarias and Sardines. The total incidence of *L. monocytogenes* in raw examined fish was 9.3%. *L. monocytogenes* was predominated in Clarias (4.19%) followed by

Saurus (2.79%) then Sardines (2.33%). In relation to the total incidence, the occurrence of *L. monocytogenes* differed in the examined fish species as it was higher (12.86%) in Clarias and lower incidence was observed in Sardines (7.69%) then Saurus (7.5%) as shown in Table (1).

Many investigators recorded the incidence of *L. monocytogenes* in raw fish. Higher values were recorded by Hartemink and Georgsson (1991) who isolated *L. monocytogenes* from 25.7% of raw fish samples while Bianchini *et al.* (1999) and Hoffman *et al.* (2003) recorded 34% and 14.6%. On the other hand, lower values were recorded by kwiatek (2004), Thimothe *et al.* (2004) and Markkula *et al.* (2005) as the incidence in raw fish was 1.26%, 3.8% and 4%, respectively. In contrast, Baek *et al.* (2000) reported that no *L. monocytogenes* was found in salt water fish and Handa *et al.* (2005) never isolate *L. monocytogenes* from raw fish. Our results are in accordance to large extent with Erdenling *et al.* (2000) who found certain type of *L. monocytogenes* associated with cat fish fillets might be a risk of listeriosis for human and Naser (2003) who recorded the presence of *L. monocytogenes* (8%) in Saurus *spp*, This variation in results may be attributed to fish species, transport, purchased places, country variation and hygienic measures or seasons.

Only limited data are available on the growth characteristics of *L. monocytogenes* in naturally contaminated raw fish, so Table (2) deals with characterization of 20 strains of *L. monocytogenes* isolated from some local raw fish. Carbohydrate profiles showed variation in sugar utilization of dalcite, lactose, maltose, mannitol, starch and xylose, while glucose, salicin, sucrose, trehalose and rhaminose yielded positive in all strains. This result is in accordance to Margolles *et al.* (2000) who found that all isolates of *L. monocytogenes* fermented L. rhammose and mannoside, while Hitchins (2003) recoded that *L. monocytogenes* fail to utilize xylose and was positive to rhaminose utilization. Van Coillie *et al.* (2004) depended on haemolycin and mannitol, rhaminose and xylose utilization test in identification and characterization of *L. monocytogenes*.

Also, tolerance of *L. monocytogenes* to NaCl that grew at high salt concentrations ranged from 8% to 20% was observed. The results in the present study are in accordance with Guyer and Jemmi (1991) and Margolles *et al.* (2000) who found that the minimal inhibition concentration was between 9% and 12% at 37°C for *L. monocytogenes* growth. Liu *et al.* (2005) recorded that *L. monocytogenes* strains tested were resistant to saturated NaCl (40 W/V) for a long time. It is clear

from this result that high light salt treatment is not sufficient to eliminate *L. monocytogenes* from salted food products.

It is obvious form Table (2) that the growth inhibition of *L. monocytogenes* in presence of organic acids did not occur within 5 and 10 minutes but occurred after 24 h incubation at 37°C (even with different concentrations 0.5, 1, 2 and 5%). There were no differences in the effect among organic acids used for growth inhibition of *L. monocytogenes*. Many authors concerned with the effect of organic acids on growth of *L. monocytogenes* as Young and Foegeding (1993) who found that when the initial pH values of acetic and lactic acids decreased or the total acid concentration increased the growth rates of *L. monocytogenes* decreased, the relative inhibition effect was generally acetic > lactic > citric. Margolles *et al.* (2000) recorded that the inhibitory capability of organic acids followed the order citric> lactic>acetic high level and high concentration being more inhibitory than low concentration. While Gravesen *et al.* (2004) said that all *Listeria* strains were sensitive to D -lactic acid which gave 0.6-2.2 log unit greater reduction than L- lactic acid midway in the activation curves

Positive Congo red binding activity and Lecithinase production were 90% and 85%, respectively. Many authors concerned with Congo red and Lecithinase production as Nunes and Hofer (1994) who evaluated Lecithinase production and capacity for Congo red absorption, presented 51.8% and 88.8% positively rates respectively. Whereas Delgado-da-Silva *et al.* (2001) recorded that the percents were 27.5% and 12.6% respectively.

The drug susceptibility is one of the important factors of characterization of *L. monocytogenes*. It is clear that all isolates were resistant (100%) to colistin sulphate, enrofloxacin, nalidixic acid and oxolinic acid, but all strains were susceptible to chloramphenicol (100%) so is considered the antibiotic of choice, followed by erythromycin (80%), amoxycillin (75%) then oxytetracycline (70) and ciprofloxacin (65%).

Lowest number of isolates were sensitive to the remaining studied antibiotics. These results indicated an increasing incidence of antibiotic resistant of *L. monocytogenes*. This could be attributed to the fact that *Listeria* may have plasmid encoding antimicrobial resistance. Also, Vaz-Velho *et al.* (2001) recorded that 190 isolates of *L. monocytogenes* isolated from fresh and cold smoked fish were susceptible to tetracycline. Poyart- Salmeron *et al.* (1990) and Hadorn *et al.* (1993) concluded that some strains of *L. monocytogenes* contain

plasmid encoding resistance to choramphenicol, erythromycin tetracycline and streptomycin, while McLauchlin *et al.* (1997) suggested that resistance to tetracycline may be useful as an easily recognized epidemiological marker for *L. monocytogenes* .

Table (2) clears that all 20 strains could cause keratoconjunctivities in G. pig and only 16 strains killed the mice within 7 days. The obtained results coincided to large extent with Nunes and Hofer (1994) who recorded the ability of *L. monocytogenes* to produce keratoconjunctivitis in G. pigs and Hitchins (2003) who found that 10^4 CFU pathogenic *L. monocytogenes* caused death usually within 3 days in mice. The obtained results disagree with Takeuchi *et al.* (2003) who observed no deaths of mice inoculated with 10^8 CFU ,but (20%) to (40%) of mice died when inoculated with 10^9 CFU of *L. monocytogenes*.

Concerning Table (3), it is clear that all strains were haemolytic (100%) to RBCs of horse, sheep, G. pig and human. Only 85, 75, 50 and 50% showed change in haemolysis with addition of D-mannose in horse, sheep, G. pig and human respectively. Also, there were variations in C.H.U. and M.H.U. between strains and species of used RBCs as high concentration (titre) occurred in sheep followed by G. pigs then human RBCs as shown in Table (4). Our results are in accordance with Margolles *et al.* (2000) who found that *L. monocytogenes* displayed haemolytic activity to a variable extent depending on the strain C.H.U between 0 and 6 while M.H.U between 12 and 96. The synthesis of haemolysin was correlated with the virulence due to its cytotoxic for leucocytes.

All strains of *L. monocytogenes* recovered from fish appeared to contain most pathoegnic and virulence factor. The trails to control *L. monocytogenes* in raw fish including heat treatment were shown in Table (5). There was direct relation between time of exposure and degree of temperature on count of *L. monocytogenes* in Sardines. It was clear that increasing time of exposure and degree of temperature reduced count as 100°C for 20 minutes completely eliminated *L. monocytogenes*, while at 70°C for 20 minutes only reduced count. On the other hand, 50°C had no effect on count even with increasing time. Our results agreed to some extent with Autio *et al.* (1999) who recorded that use of hot steam, hot air and hot water seemed to be useful in eliminating *L. monocytogenes*, while Thimothe *et al.* (2002) found that heat treatment significantly reduced *L. monocytogenes* contamination of RTE craw fish meat. Naser (2003) recorded that fish fillets samples that subjected to

grilling for 6 and 8 minutes gave 4 X 10^4 and 1.3 X 10^3 CFU/ g respectively.

The counts of *L. monocytogenes* (Table 6) inoculated in Sardine were affected by treatment with organic acids in used concentrations as the acid concentration increased the growth rates of *L. monocytogenes* decreased. The relative inhibition effect was generally lactic > acetic > citric acid. The obtained results coincided with that of Zeitoun and Debevere (1991) who found that the antimicrobial effect of lactic acid buffer systems pH 3.0 increased with increasing concentration of lactic acid in the buffer system on chicken leg. Also Pothuri *et al.* (1996) studied the effect of lactic acid on growth and survival of *L. monocytogenes* in cry fish at concentration of 0.5, 1, 1.5 and 2% and found that count declined steadily with 2% lactic acid. Naser (2003) said that citric acid was less effective than acetic acid in controlling *L.monocytogenes* in fish fillet samples

It is of interest in this work that low concentration of organic acids (0.5%) enhanced growth of *L. monocytogenes* and this is in accordance with Pothuri *et al.* (1996) who found that stimulations of growth by 0.5% lactic acid may be due to utilization of lactic acid as a carbon and energy sources. Our results indicated the importance of choosing an adequate concentration of organic acids used for the decontamination of fish because low concentration could favour the growth of *L. monocytogenes*.

The inhibitory action of acetic acid is produced through utilization of electrochemical ingredient of the cell membrane as well as denatutrating protein inside the cell but the microbial effect of citric acid is partially due to its ability to chelate divalent cations, while lactic acid penetrate the cell membrane and act by releasing a proton and acidifying the cytoplasm of the cell.

It is clear from Table (7) that application of antimicrobial ice in commercial concentration on fish not completely eliminated but reduced *L. monocytogenes* count from 1 X 10^8 to 1.5 X 10^5 and 2 X 10^3 after 60 and 120 min exposure respectively.

This is in agreement with Kim *et al.* (1999) who reported that treatment with antimicrobial ice up to 200 ppm slightly reduced the number of mesophilic bacteria. Also Shin *et al.* (2004) recorded that the use of antimicrobial ice (100 ppm) on fish skin for 120 min, only reduced count of *L. monocytogenes*. There is difference with the study conducted by Huang *et al.* (1997) who found that the amount of antimicrobial ice required to destroy (95%) of gram +ve and gram –ve

bacteria was 1.3 and 1.18 ppm respectively. This may be attributed to nature of strains and country difference. Also antimicrobial ice can be easily used on seafood products as generally stored and displayed on ice, also maintain a constant level of ClO_2 gas during melting.

From the results of the present work, it can be concluded the importance of regular *L. monocytogenes* testing of raw fish and improve microbiological quality, good hygienic measure and continuous disinfection of utensil, shops and markets. We suggest the inactivation treatments that effectively eliminate *L. monocytogenes* present in raw fish by addition of lactic or acetic or citric acid to fish during eating and / or good grilling of fish. A need of further studies of sensory quality (appearance odour freshness- texture and freshness of taste) must be taken in consideration.

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