

### Bacteriological investigation on *Listeria monocytogenes* in Egyptian food samples with special reference to its resistance patterns

Submitted in partial fulfillment of Master's degree in Pharmaceutical Sciences (Microbiology and Immunology)

Presented by Rasha Ibrahim Mohamed El Shamy BSA in Pharmaceutical science, (2009) Faculty of Pharmacy, Cairo University

Under supervision of

#### Prof. Ramy Karam Aziz

Professor and Chair, Department of Microbiology and Immunology, Faculty of Pharmacy, Cairo University

#### Dr. Mohamed Abdalla Abd El Moneam

Associate professor and Chair, Department of Microbiology, Central Laboratory of Residue Analysis of Pesticides and Heavy Metals in Food, Agriculture Research Center

> Department of Microbiology and Immunology Faculty of Pharmacy Cairo University 2019

## TABLE OF CONTENTS

Contents	Page	
INTRODUCTION AND AIM OF WORK	1	
LITERATURE REVIEW	3	
1.History of <i>Listeria</i>	3	
2. Prevalence of <i>L. monocytogenes</i> in different types of food	3	
3. Genome of <i>L. monocytogenes</i>	4	
4. Cell structure	5	
5. Pathogenic <i>Listeria</i> spp.	5	
6. Incidence of illness and outbreak data		
7. Survival and mode of transmission of <i>L. monocytogenes</i>	7	
8. Pathogenesis of <i>L. monocytogenes</i>	8	
9. Virulence factors	9	
9.1 Internalins	9	
9.2 Listeriolysin O	9	
9.3 Act A protein	10	
9.4 Phospholipases	10	
9.5 Protein p 60	10	
10. Serogrouping of L. monocytogenes	10	
11.Diagnosis	11	
12.Treatment and prevention	12	
13.Antibiotic sensitivity	14	
MATERIALS AND METHODS		
1. Materials	15	
a. List of media	15	
1. Enrichment media		
a. Fraser Broth Base	15	
a. Half Fraser Broth primary enrichment	15	
1. Half Fraser selective supplement	15	
b. Fraser Broth secondary enrichment	15	
1. Fraser selective supplement	16	
2. Identification and Isolation media	16	
a. Chromocult® Listeria Agar Base according to Ottaviani and	16	
Agosti (ALOA)		
1. Supplements of ALOA	17	
b. <i>Brilliance Listeria</i> Agar	17	
1. Brilliance Listeria selective supplement	17	
2. Brilliance Listeria differential supplement	18	
3. Media used for Antibiotic sensitivity test		
a. Muller Hinton agar	18	
3. Biochemical Kits microbact 12L	18 19	
4. Reagents and chemicals		

Contents	Page	
5. Reference strains	19	
6. primers and probes		
7. Real Time PCR		
8. Antibiotic susceptibility	19	
9. Equipment	20	
2. Methods		
a. Sample collection	21	
b. Isolation of <i>L. monocytogens</i>	21	
c. Confirmation by biochemical test (Micobact 12 L)	21	
d. Preservation of confirmed isolates Bacterial preserver.	22	
e. Confirmation of L. monocytogenes isolates using Real time	22	
PCR.		
1. Extraction of DNA	22	
2. Real time PCR mixture solution	22	
3. Real time PCR Runs	23	
F .Detection of virulence factors	23	
G .Antibiotic susceptibility test	24	
H. Antibacterial activity of herbs extract	26 27	
RESULTS		
1. Distribution of <i>L. monocytogenes</i> in frozen food samples.	27	
2. Distribution of <i>L. monocytogenes</i> in spices and herbs.	29	
3. Conventional identification of <i>Listeria</i> .	29	
4. Morphological and biochemical confirmation of <i>L</i> .	30	
monocytogenes.		
5. Phenotypic determination of hemolytic activity of <i>L. monocytogenes</i>	31	
6. Detection of virulence gene profile of <i>L. monocytogenes</i> by real time PCR	34	
7. Susceptibility of the L. monocytogenes isolates to antibiotics	35	
8. Statistical analysis	39	
9. Antibacterial effect of four herbs extract against <i>L. monocytogenes</i>	39	
isolates		
DISCUSSION	41	
CONCLUSIONS	46	
REFERENCES	47	
SUMMARY	63	
الملخص العربي المستخلص العربي	-	
المستخلص العربي	-	

Fig. No.	Title	Page
1.	The amplification curves for the positive samples using PIKO 96 Thermo Scientific Real Time PCR.	23
2.	Distribution of isolated <i>L. monocytogenes</i> among vegetable samples	28
3.	Distribution of isolated <i>L. monocytogenes</i> among fruit samples.	28
4.	Isolation of <i>L. monocytogenes</i> on ALOA .Blue-green colonies surrounded by opaque halo zone appeared on ALOA agar.	29
5.	Identification of <i>L. monocytogenes</i> on Chromogenic Agar characteristic blue-green colonies surrounded by opaque halo zone is shown.	29
6.	Gram-positive coccobacillary rods of L. monocytogenes	30
7.	Identification of L. monocytogenes by Microbact 12L.	30
8.	Hemolytic activity among L. monocytogenes isolates	32
9.	$\beta$ hemolysis of <i>L. monocytogenes</i> on sheep blood agar	33
10.	Amplification of the iap (171bp) gene for samples positive for <i>listeria monocytogenes</i> .lane 1:100-bp DNA ladder marker, lane 2-9: samples positive for <i>iap</i> gene.	34
11.	amplification of the <i>hly</i> (162bp) gene for samples positive for <i>listeria monocytogenes</i> .lane 1:100-bp DNA ladder marker, lane 2-9: samples positive for <i>hly</i> gene.	34
12.	Percentage of sensitivity and resistance against 47 <i>L. monocytogenes</i> isolates	35
13.	Plates showing results of a representative antibiotic susceptibility test for <i>L. monocytogenes</i> isolates.	36

### LIST OF FIGURES

# LIST OF TABLES

Table No.	Title	Page
1.	list of listeriosis outbreaks	6
2.	Limits for growth of <i>L. monocytogenes</i>	8
3.	CDC recommendations for food handling and storage to	13
	prevent listeriosis	15
4.	Ingredients of Fraser broth base	15
5.	Ingredients of ALOA agar base	16
6.	Ingredients of Chromogenic agar medium	17
7.	Mode of action of media used for identification of	18
	L.monocytogenes	
8.	Ingredients of Muller Hinton agar	18
9.	Substrates of Microbact 12L system used to identify L.	19
	monocytogenes.	19
10.	List of chemicals and reagents and its sources	19
11.	List of Antibiotic disc	20
12.	List of Instruments	20
13.	Reactions of Microbact 12L	22
14.	Primers used in RTi-PCR assays for two virulence	24
	genes of L. monocytogenes	24
15.	Primers sequences. Expected amplicon sizes and	24
	annealing temperatures.	
16.	Interpretation of zone diameter of inhibition among the	25
	antimicrobial agents	23
17.	Prevalence of L. monocytogenes among frozen samples	27
18.	Reactions of positive samples of L. monocytogenes in	31
	Microbact 12L system.	-
19.	Hemolytic activity of 47 Isolates.	32
20.	Percentage of sensitivity and resistance against 47 L.	36
	monocytogenes isolates.	50
21.	susceptibility of 47 L. monocytogenes isolates to 10	
	antibiotics determined by Disk diffusion in (mm) and	37
	hemolytic activity	
22.	Antibacterial activity of spearmint, ginger, garlic and	40
	Nigella sativa extracts on isolates of L. monocytogenes	υF

# LIST OF ABBREVIATIONS

AK	Amikacin
ALOA	Agar Listeria according to Ottaviani and Agosti
AML	Amoxicillin
CAL	Ceftazidime + clavulanic acid
CDC	Centers for Disease Control and Prevention
CFU	Colony-forming unit
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
CN	Gentamicin
Ct	Cycle threshold
DNA	Deoxyribonuceic acid
Ε	Erythromycin
EFSA	European Food Safety Authority
HACCP	Hazard analysis and critical control point
<i>hlyA</i>	Hemolysin gene
IAC	Internal Amplification Control
Іар	Invasion-associated protein
InlA	Internalin A
InlB	Internalin B
LIPI-1	Listeria pathogenicity island
LLO	Listeriolysin O
MPN	Most probable number
NCCLS	National committee for clinical and laboratory standards
NOR	Norfloxacin
PC-PLC	Phosphatidylcholine-specific phospholipase c
PCR	Polymerase chain reaction
PG	Penicillin G
PI-PLC	Phosphatidylinositol-specific phospholipase c
<b>PrfA</b>	Positive regulatory factor
RTE	Ready-to-eat food
SXT	Trimethoprim/ sulfamethoxazole
C30	Chloramphenicol

#### ABSTRACT

Listeria monocytogenes is among the most important foodborne pathogens. It may enter food processing environments through raw materials, handlers or equipment and may persist due to ineffective cleaning or sanitation. The bacterium can be isolated from both frozen vegetables and fresh food substances. This study aimed to estimate the prevalence of L. monocytogenes in spices and frozen vegetables and screen for some virulence factors and drug-resistance determinants of the isolated bacteria. First, conventional microbiological methods were used for the isolation and identification of bacteria. Next, the identity of isolated bacteria was confirmed by molecular techniques, and the virulence genes *iap* and *hlvA* were identified by real-time polymerase chain reaction (PCR). The hemolytic activity of the isolates was assessed by cultivation on sheep blood agar. Furthermore, the antimicrobial susceptibility of confirmed L. monocytogenes isolates was tested by the disk diffusion method against 10 antibiotics. Out of 331 vegetable samples, 47 isolates were confirmed to contain L. monocytogenes, whereas none of 40 spice samples tested positive. All isolates were positive for *iap* and *hlvA* genes. Susceptibility testing indicated that all isolates were sensitive to trimethoprim/sulfamethoxazole, but only 36% were sensitive to penicillin G, while 100% and 70% showed intermediate resistance to chloramphenicol and erythromycin, respectively. All tested isolates were resistant to amoxicillin, gentamicin and norfloxacin; on the other hand, 90, 86 and 84% of the tested strains were resistant to ciprofloxacin, ceftazidime/clavulanic acid and amikacin, respectively. In summary, L. monocytogenes isolates disseminated in frozen vegetable samples from the Egyptian market were highly virulent, entirely multiple-drug resistant and were enriched in iron-containing vegetables. Since L. monocytogenes is primarily pathogenic to humans and causes a life-threatening disease, there is a potential infection risk for people who usually deal with frozen vegetables before cooking. Hence, surveillance to L. monocytogenes in frozen products, together with implementation of tight measures would be valuable in preventing listeriosis, and are highly recommended.

Key words: Listeria monocytogenes- virulence genes- antibiotic resistance.