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Assessment of efflux pump genes expression of listeria monocytogenes isolated from diarrhea patients using qRT-PCR technique

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Abstract

Efflux pump genes are an important resistance mechanism that may be specific for one substrate or may transport a range of structurally dissimilar compounds. Efflux pumps represent an important and large group of transporter proteins found in almost all organisms. In this study, potential mechanisms underlying resistance of Listeria monocytogenes to humans' intestinal bile were investigated. These results indicated that listeria monocytogenes efflux pump genes have a critical role in the resistance and survival of this pathogen to human bile and other antibacterial components. The efflux pumps reside in their ability to extrude a wide range of antibiotics, resulting in the emergence of multidrug resistance in many bacteria. Besides antibiotics, efflux pumps can also extrude a large variety of compounds.

Keywords: Efflux pumps genes, listeria monocytogenes, diarrhea patients, qRT-PCR technique, human bile.

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Introduction

Listeria monocytogenes is a facultative anaerobic bacterium, which has the ability to survive in the presence or absence of oxygen. Which can grow and multiply inside the host's cells and is one of the most lethal foodborne pathogens: 20-30% of foodborne listeriosis infections are in individuals at high risk of infection and may be fatal.[1-3] Responsible for an estimated 1,600 illnesses and 260 deaths in the United States annually, listeriosis ranks third in the total number of deaths among foodborne pathogens.

Listeriosis follows a trend of escalation in the number of infections. The death rate of listeriosis is higher in the European Union than other foodborne pathogens [1-4]. Also, listeriosis is a serious infection caused by the bacterium Listeria monocytogenes. People usually get listeriosis after eating contaminated food. The disease affects pregnant women, newborns, the elderly, and people with weakened immune systems. Listeria bacteria can survive during refrigeration and even freezing. Therefore, people at high risk of serious infections should avoid eating foods that are likely to contain listeria bacteria. Listeria monocytogenes is widely distributed in the environment and can cause serious human infections such as Rheumatoid arthritis CNS, especially in neo/nates and immunocompromised adults [5]. Abortion The transmission of infection through food is recognized as the main route. The acquisition of infection during the various epidemiological regulations. Also, it is a Gram-positive bacterial factor that causes major deaths and diseases. The ability to invade both phagocytic and non-phagocytic cells and replicate within the cytoplasm of infected cells [6] is crucial to causing intracellular proliferation. Contaminated food, especially ready-to-eat foods, is a major transmission route to humans [7]. Irrigation water and agricultural soil also contain L. monocytogenes that are multidrug resistant and are likely to be distributed to fresh agricultural products and pose a food safety risk [8]. Poor hygiene and inadequate implementation of standard sanitation operating procedures (SSOPs) have led to an outbreak of listeriosis [9]. Moreover, pathogens will easily form.The adaptation of the pathogen to environmental stresses and adaptation that may occur before exposure to sub-lethal concentrations of antimicrobial agents has contributed to antimicrobial resistance [10,11]. Environmental conditions: The use of this pathogen is a vital matter and its control and the development of new factors, a substance for microbes against it. Merely or when consumed, the pathogen is capable of traversing epithelial barriers, cellular invasion and intracellular replication when modulating virulence factors such as endogenous and hemolysin. Potentially agronomic agents (plasmids and transposons) and other complex mechanisms [12]. Flux pumps are transport proteins that are involved in the extrusion of toxic substrates (including all classes of clinically relevant antibiotics) from within cells to the external environment. These proteins are found in both Gram-positive and Gram-negative bacteria as well as in eukaryotic organisms.

Cell membrane changes are thought to be responsible for this non-specific increase in resistance. Perhaps efflux pumps, another important resistance mechanism, are specific to a single substrate or may transport a group of structurally dissimilar compounds [13]. These pumps can be associated with

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multiple drug resistance. Broadly, most genes encoding multiple drug transporters may be located on the chromosome, although some are reported to be plasmid-mediated [14]. It has been hypothesized that multidrug-resistant efflux pumps were selected by bacteria to avoid the effects of toxic compounds present in their natural environment.

L.monocytogenes efflux pumps contain a number of genes including MdrL, Lde, MdrT, MderM, MarR, and lde gene (Listeria drug efflux; formerly lmo2741) encodes a 12- transmembrane-segment putative efflux pump belonging to the major facilitator superfamily of secondary transporters that displayed 44% identity with PmrA from Streptococcus pneumoniae. Insertional inactivation of the lde gene in CLIP 21369 indicated that the protein was responsible for fluoroquinolone resistance and was involved in the level of susceptibility to dyes such as ethidium bromide and acridine orange.

L. monocytogenes isolates from human cases of listeriosis in France. On the basis of a fourfold or greater decrease in the ciprofloxacin MIC in the presence of reserpine, fluoroquinolone resistance was attributed to the active efflux of the drugs. The lde gene (Listeria drug efflux; formerly lmo2741) encodes a 12-transmembrane-segment putative efflux pump belonging to the major facilitator superfamily of secondary transporters that displayed 44% identity with PmrA from Streptococcus pneumoniae. Insertional activation of the lde gene in CLIP 21369 indicated that the corresponding protein was responsible for fluoroquinolone resistance and was involved in the level of susceptibility to dyes such as ethidium bromide and acridine orange.

Flow pumps are recognized as an important mechanism for decreasing sensitivity to benzalkonium chloride (BC) in Listeria monocytogenes. Previous studies showed that the MdrL efflux pump was overexpressed in L.monocytogenes exposed to BC.

In the present work, we aimed to elucidate the role of MdrL in tolerance of BC and environmental stresses including acidic, alkaline, osmotic, ethanol and oxidative stresses, as well as resistance to other antimicrobial agents in L. monocytogenes EGD-e.

In addition, the regulation of mdrL expression by LadR was investigated. Gene deletion mutants were generated by the homologous recombination strategy. For wild-type and mutant strains, minimum inhibitory concentrations (MICs) of antimicrobial agents were determined by agar dilution, and growth and survival analysis were also performed. LadR was expressed and the interaction between LadR and the mdrL promoter was investigated by electrophoresis shift assay (EMSA).

Compared with the wild-type strain, the growth of the ΔmdrL deletion mutant strain is impaired in the presence of a sub-lethal concentration of BC. Moreover, the mutant showed a lower survival level than the wild-type strain in the presence of a lethal concentration of BC. However, mdrL deletion had no effect on cefotaxime resistance and ethidium bromide efflux.BC could induce the expression of mdrL in L. monocytogenes and mdrL expression was regulated by LadR rather than SigmaB. LadR was able to specifically bind to the mdrL promoter. The results showed that the MdrL efflux pump was associated with BC tolerance in L. monocytogenes EGD-e. Moreover, our results also provided strong evidence that LadR negatively regulates the expression of mdrL. Since BC is commonly used in the food industry,

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The MdrL efflux pump is useful for L. monocytogenes to overcome this stress in food processing environments. However, MdrM and MdrT belong to the major facilitator family (MFS) of MDR vectors and are closely related to the well-characterized MDR vector, QacA, for Staphylococcus aureus [15]. MDR transporters, such as QacA, are well-known for their ability to confer resistance to a wide range of toxic compounds and drugs, including antibiotics, by utilizing the proton driving force to extrudate these compounds with extracellular activity [16]. Accordingly, MdrM and MdrT were shown to be transcriptionally induced upon bacterial exposure to rhodamine 6G (R6G) and tetraphenylphosphonium (TPP), both known substrates of MDRs, and confer resistance to cholic acid [17]. That is, none of the classical MDR functions can explain the observed role of these proteins in the activation of the innate immune system, implying that they may have distinct physiological roles during infection.

It was recently suggested that MdrM and MdrT vectors extrudate cyclic-di-AMP(c-di-AMP) during intracellular growth of L.monocytogenes, which in turn activates infected macrophages to elicit an IFN-γ response [18,19].

One significant family of such transcription factors is the Multiple antibiotic resistance Repressor (MarR) family. Members of this family are well conserved across different bacterial species and in some cases, are known to regulate vital bacterial functions. This review focuses on the role of MarR family transcriptional factors in antibiotic resistance within a select group of clinically relevant pathogens. MarR homologs regulate the activity of genes involved in stress responses, virulence, or degradation or export of harmful chemicals such as phenolic compounds, antibiotics, and common household detergents.

The MarR family is identified as a group of regulatory factors whose activity is modulated in response to environmental signals in the form of phenolic compounds. Many of these agents are plant derived. Some of the MarR homologs appear more likely to control systems expressed in animal hosts, suggesting that phenolic sensing by bacteria is important in a variety of environments and in the regulation of numerous processes.117.

Materials and Methods

- **Equipment and instruments**

The laboratory instruments and equipment, culture media, chemicals, commercial kits and primers used in this study are illustrated in tables (1), (2), (3), (4), (5), respectively.

Table 1. The instruments and equipment used in this study with their remarks.

No.	Instrument	Company/ Country of Origin
1.	**Autoclave**	**Mammert/Germany**
2.	**Benson Burner**	**Mammert/Germany**
3.	**Camera**	**Canone/Japan**
4.	**Centrifuge (OHAUS)**	**Hettich, Germany**
5.	**Disposable Petri dishes**	**Al-Hani company / Lebanon**

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6.	**Electric Oven**	**Mammert/Germany**
7.	**Electrophoresis**	**Shando, scientific co. / UK**
8.	**Eppendorf tubes**	**Sterellin Ltd. / UK**
9.	**Hot plat stirrer**	**Labtech /Korea**
10.	**Incubator**	**Mammert/Germany**
11.	**Inoculating loop**	**Japan**
12.	**Light Microscope**	**Olympus/Japan**
13.	**Micropipettes 5-50, 0.5-10, 100-1000µl**	**CYAN/ Belgium**
14.	**Pipette**	**Volac, U.K**
15.	**Plain tubes (10)**	**Bromed, USA**
16.	**Refrigerator(-20)**	**Concord /Lebanon**
17.	**Sensitive Balance**	**Sartorius/Germany**
18.	**Standardized loop**	**Himedia, India**
19.	**Sterilized cotton swabs**	**Sterile EO. / China**
20.	**Thermocycler PCR**	**MWG Biotch /Germany**
21.	**Vortex**	**CYAN/ Belgium**
22.	**Water Bath**	**Mammert/Germany**
23.	**UV gel documentation**	**GFL-Germany**
24.	**Real time PCR system bio-GENER (Q3200)**	**China**
25.	**High speed Cold Centrifuge (OHAUS)**	**Hettich, Germany**
26.	**VITEK2Compact**	**United States of America**
27.	**Fluorescence microscopy**	**USA**
28.	**Hood**	**Gellman, England**

- **The Chemicals**

Table 2. Chemicals and Solutions used in the study

No.	**Chemicals**	**Company**	**Country**
1	Chloroform	Labort	India
2	DEPC water	Bioneer	Korea
3	Isopropanol	Labort	India
4	Ethanol 100%	Labort	India
5	RNase free water	Bioneer	Korea
6	Trizol reagent 100ml	Bioscience	USA
7	DNase I enzyme	Promega	USA

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8	B-mercaptoethanol	Reagent World	USA
9	10% formalin	Xinlongwel	China

Table 3. Cultural media that used in this study with their remarks.

No.	Culture media	Company / origin
1	Blood Agar (BA) Base	Mumbai (India)
2	Brain Heart Infusion Broth (BHIB)	
3	MacConkey agar (MA)	
4	Nutrient Agar (NA)	
5	Nutrient Broth (NB)	

- **kit**

Table 4. The kits used in this study with their remarks.

No.	Kit	Company	Country
1	Presto™ Mini gDNA Bacteria Kit	Geneaid	Taiwan
2	GoTaq® Green PCR master Mix	Promega	USA
	GENEzol™ TriRNA Pure kit	Geneaid	Korea
	AccuPower^R RocketScript™ RT PreMix(cDNA synthesis)	Bioneer	Korea
	AccuPower^R Green star™ qPCR PreMix (SYBER Green)	Bioneer	Korea

- **primer**

Table 5. The primers used in this study and their product size designer, sequencing, purpose, accession number, and Provider Company.

Target gene	Primer sequence (5’- 3’)		Product size (bp)	ACCESSION No.	Company/ Country
Lde	F	GATTCTGATGGGACTCGTAAGC	22	MG673928	Scientific Researcher. Co. Ltd in Iraq
	R	CGATGACGAGGAACTTGTGTAG	22		
	F		22		

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MdrL		**TGGACCACTACTAGGAGGTATT**	**21**	**MG673927**	**Scientific Researcher. Co. Ltd in Iraq**
	R	**GCGACTTTCTTCGGTTCTTTC**			
MdrM	**F**	**CAACGCGTCAGCTTTACTTATTC**	**23**	**AB671771**	**Scientific Researcher. Co. Ltd in Iraq**
	R	**GCACCAATTGCTTGTACCATAC**	**22**		
MdrT	**F**	**GCAATGATTGGTGCGGAAAT**	**20**	**AB671767**	**Scientific Researcher. Co. Ltd in Iraq**
	R	**ATCGCACCCGGAAGTAATAATAG**	**23**		
MarR	**F**	**GCGCGCTCAGCTTAGAATTA**	**20**	**AB671770**	**Scientific Researcher. Co. Ltd in Iraq**
	R	**TTTAACCGATCCTCGCCAATC**	**21**		

- **Methods**

The design of study

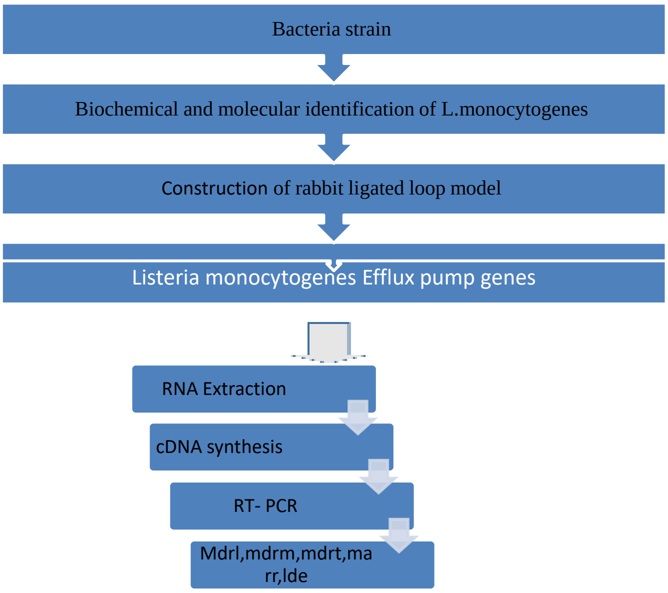

Figure 1. The design of the study

- **Culture Media:**

Culture media for identification and characterization of L.monocytoges:

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All media were prepared according to manufacturing company instructions after they were heating on a benzene burner, dissolve the constituents completely and then autoclaved, they were sterilized at 121 C (15 Ib/inch2) for 15 min. Then, the media were incubated at 37 C for 24 hours to ensure sterilization, contaminated media were discarded. The media identification of L.monocytoges was done by using the following media:

1. Blood agar media:

Prepared by dissolving 40 gram of blood agar base dissolve in I L distilled water

2. Nutrient broth

Prepared by 13 gm of nutrient broth in 1 litter of distilling water

3. Brain-Heart Infusion (broth)

It was prepared by dissolving 34.5 gm in a liter distilled water. BHI used to keep the bacteria survive in broth culture at (0□C to 4□C) after mixed with 15% glycerol (85 ml brain-Heart infusion with 15 ml glycerol).

- **Listeria monocytogenes Strains**

was obtained from the laboratory of Sheikh Zayed Hospital in Baghdad, and then transferred to the College of Biotechnology Laboratory at Al-Qasim Green University. After that the bacteria were cultured on routine culture media characteristics were studied depending on colony morphology (colour, size, consistency, density) on the growth media, also the type of hemolysis (α, β or γ hemolysis) on blood agar. and confirmed diagnosis again by:

- **The Vitek 2 analyzer for routine bacterial identification**

The identification of the L.monocytogens by Vitek 2 Gram-Positive analyzer automated system was done in Al-Safwa Specialized Laboratory in Babylon/ Iraq to confirm the diagnosis of L.monocytogen. The VITEK® 2 GP identification card (GP) is intended for use with VITEK® 2 Systems for the automated identification of most significant Gram-positive bacteria is based on 64 biochemical tests measuring carbon source utilization, inhibition and resistance, and enzymatic activities. Identification results are available in approximately 8 h or less. Briefly, Listeria was cultured on nutrient agar for 18–24 h at 37 _C before subjected analysis.

- **Diagnosis of Listeria monocytogenes by Polymerase Chain Reaction (PCR)**

Based on [20] described the protocol of PCR primers used in the interaction and gel extraction (Sequencing) protocol (Vogelstein et al., 1979) .

PCR technique was performed for molecular detection of based Listeria monocytogenes protein based 16S ribosomal RNA gene . PCR primers that used in these techniques were designed in this study and done as following steps

A. Bacterial DNA extraction

Bacterial genomic DNA was extracted bacterial isolates by using (Presto™ Mini gDNA Bacteria Kit) as and done according to company instructions as following steps:

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1-Sample Preparation :

1 ml incubated cultured bacterial cells (up to 1 x 109) was transferred to 1.5 ml microcentrifuge tube then centrifuged at 10000 rpm for 1 minute then the supernatant was discarded.

2-Cell Lysis steps :

a- 180µl GT buffer was added to the tube and the cell pellet was suspended by vortex, then 200µl of Lysozyme buffer was added and the mixtures were incubated at 60ºC for 10 minutes. During incubation periods, the mixtures tubes were inverted every 3 minutes.

b- 200µl GB buffer was added to each tube and mixed by vortex for 10 seconds. Then the tubes incubated at 60ºC for 10 minutes with inverted tubes every 3 minutes through incubation periods.

3-DNA binding steps :

a- 200µl absolute ethanol was added and immediately mixed by vortex, then precipitates if happen was broken by pipetting.

b- A GD column was placed in a 2 ml collection tube and all mixtures were transferred (including any precipitate) to the GD column. Then centrifuged at 10000 rpm for 1 minute. The 2 ml collection tubes that contain the flow-through were discarded and placed in the GD column in a new 2 ml collection tube.

4-Washing steps :

a- 400µl W1 buffer were added to the GD column, then centrifuged at 10000rpm for 1 minute. The flow-through was discarded and placed the GD column was back in the 2 ml collection tube.

b-600µl Wash Buffer was added to the GD column. Then centrifuged at 10000 rpm for 1 minute. The flow-through was discarded and placed the GD column back in the 2 ml collection tube and the tubes were centrifuged again for 2 minutes at 12000 rpm to dry the column matrix.

5-Elution steps :

a- The dried GD column was transferred to a clean 1.5ml microcentrifuge tube and 100µl of pre-heated elution buffer were added to the center of the column matrix.

b- The tubes were let stand for at least 3 minutes to ensure the elution buffer was absorbed by the matrix. Then centrifuged at 10000 rpm for 1 minute to eluted the purified DNA.

B. Estimation of extracted total DNA:

The extracted total DNA was checked by using Nanodrop (Thermo Scientific NanoDrop Lite UV. USA) that measured DNA concentration (ng/µL) and checked the RNA purity at absorbance (260 /280 nm) as following steps:

1. After opening up the Nanodrop software, choose the appropriate application (Nucleic acid, DNA).
2. A dry wipe was taken and cleaned the measurement pedestals several times. Then, carefully pipetted 2µl of free nuclease water and placed onto the surface of the lower measurement pedestals for blank the system.
3. The Nanodrop sampling arm was lowered and 1µl DNA sample was measured.

C. PCR preparation

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1- PCR master mix preparation

PCR master mix reactions for all genes was prepared by using (GoTaq ®Green PCR master kit) and this master mix done according to company instructions as following table (6)

Table 6. Standard PCR master mix protocol

PCR Master mix	**Volume**
DNA template 5-50ng	**5µL**
Forward primer (10pmol)	**2µL**
Reveres primer (10pmol)	**2µL**
GoTaq ®Green PCR master	**12.5µL**
PCR water	**3.5µL**
Total volume	**25 µL**

Then, all the PCR tubes were transferred into an Exispin vortex centrifuge at 3000rpm for 3 minutes, and then placed in T100 PCR Thermocycler (BioRad-USA).

2. PCR Thermocycler Conditions

PCR thermocycler conditions protocol for each gene was calculated by using Optima's Protocol Writer™ online application and done by using a convention PCR thermocycler.

3. PCR product analysis

The PCR products were analyzed by agarose gel electrophoresis method as following steps:

1- 1.5% Agarose gel was prepared in using 0.5X TBE and dissolved in a microwave for 5 minutes and left to cool for 50°C.

2- Then 5µl red safe e stain was added into agarose gel solution.

3- Agarose gel solution was poured in the tray after fixed the comb in the proper position and left to solidified for 15 minutes at room temperature, then the comb was removed gently from the tray.

4- The gel tray was fixed in the electrophoresis chamber and filled by 0.5X TBE buffer.

5- 10µl PCR product were loaded in to each well with added 5µl (DNA marker Ladder) in the first well. Then electric current was performed at 100 volt and 80 AM for 1 hour.

5- PCR products were visualized by using a UV Transilluminator.

- **RT-PCR preparation**

A-Sample collection and total RNA isolation:

Rabbit's intestinal loops will be isolated from the rabbits after the surgery. Total intestinal tissue RNA will be isolated. The quantity and quality of the total tissue RNA will then be analyzed using a NanoDrop.

Total RNA extraction.

Tissue

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1- A 30-50 mg tissue was placed in an Eppendorf containing 700 triazole

2- Grind a tissue homogeneously, then add 300 ul of chloroform and put it in ice for 10 minutes

3- Centrifuge for 10 minutes with a of 15000 rpm, then we take supernatant and put it in a new Eppendorf and add 300 uL lysosomes to it with a 2uL of B- mercaptoethanol.

4- Centrifuge for 5 minutes for 15,000 rpm then we take a supernatant and put it in a new Eppendorf

5- We add an equal amount of ethanol to the filtrate in the Eppendorf and mix for some time and then put it in RB column.

6-Add 400ul f wash buffer to the column then centrifuge at 15000 rpm for 30 seconds.

7- Add DNase I solution (80 ul into the center of the RB column matrix

8- Incubate the column for 15 minutes at room temperature

9- Add 400 ul of Pre – wash buffer to the RB column then centrifuge at 15000 rpm for 30 seconds and Discard the flow – through then place the RB column

10 Add 600 ul of wash buffer to the RB column

11- Add 30 ul of RNase – free – Water into the center of the column matrix

12- Centrifuge at 15000 rpm for 2 minutes to elute the purified RNA

B.cDNA synthesis:

Use the following equation to equalize the concertation of the product RNA:-

$C1\ V1 = C2\ V2$

1-Standard cDNA synthesis protocol:

Reaction mixture

component	**volume**
Template RNA	**2 µL**
Oligo dt20	**2 µL**
DEPC.D.W	**16 µL**
Total	**20 µL**

2- Perform the reaction under the following condition.

Step	**Temperature**	**Time**
cDNA synthesis	**55 c^0**	**1 hr**
Heat inactivation	**95 c^0**	**5 min**

C.RT-PCR experiments.

1- Recommended RT-PCR protocol:

component	**volume**
Forward primer (10 pmol)	**2 µL**
Reverse primer (10 pmol)	**2 µL**

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Template	**5 µL**
DEPC.D.W	**11 µL**
Total	**20 µL**

2- Perform the reaction under the following condition.

Step	**Condition**	**cycle**
Pre-Denaturation	**95 c^0,1min**	**1**
Denaturation	**95 c^0 ,5s**	**40**
Annealing/Extension		**40**
Detection (scan)	**55 c^0,40s**	**40**
Melting	**-**	**1**

3.3. Statistical Analysis:

Statistical analysis was performed using a statistical software package (IBM, SPSS V.19). T-Test was applied to detect the significant regulation (P-value <0.001).

Results and Discussion

Our results showed that all efflux pump genes of Listeria monocytogenes, Lde, MdrL, MdrM, MdrT, MarR, were significantly upregulated (P-value 0.001) compared to the control(fig. 1, 2, 3, 4 and 5). These results indicated that listeria monocytogenes use efflux pump genes to survive in the human intestine and causing diarrhea. However, Listeria monocytogenes, is a Gram-positive, facultative intracellular foodborne pathogen, and widely distributed in the environment. The frequent use of disinfectants in food environments has raised concerns about the possible role of disinfectants in cross-adaptation and facilitating the emergence of resistant isolates [21, 22,23].

Mdrm, Mdrl, mdrR, mdrT, and Lde are belong to the major facilitator superfamily (MFS) of MDR transporters efflux pumps genes group that present in listeria monocytogens. Bacterial efflux pumps are membrane proteins that can be specific for one substrate or transport a broad range of structurally dissimilar compounds from the cytoplasm to the exterior of cell [24].

Those pumps that transport several compounds can be associated with multiple drug resistance such as antibiotics, disinfectants, toxins, and dyes [25]. It is well known that increased expression of efflux pump genes can confer decreased susceptibility to antimicrobial agents [26].

In L. monocytogenes, there are two efflux pumps [22]. One efflux pump, MdrL, can extrude macrolides, cefotaxime, heavy metals, and ethidium bromide [27]. The other efflux pump is termed Lde. Although previous studies showed that efflux pump Lde is responsible for fluoroquinolone resistance and in part, for resistance to acridine orange and ethidium bromide in L. monocytogenes [28, 22, 29], little is known about the gene expression of lde in L. monocytogenes isolates kept under different conditions.

So our study considers a novel study of expression level of lde gene in L. monocytogenes.

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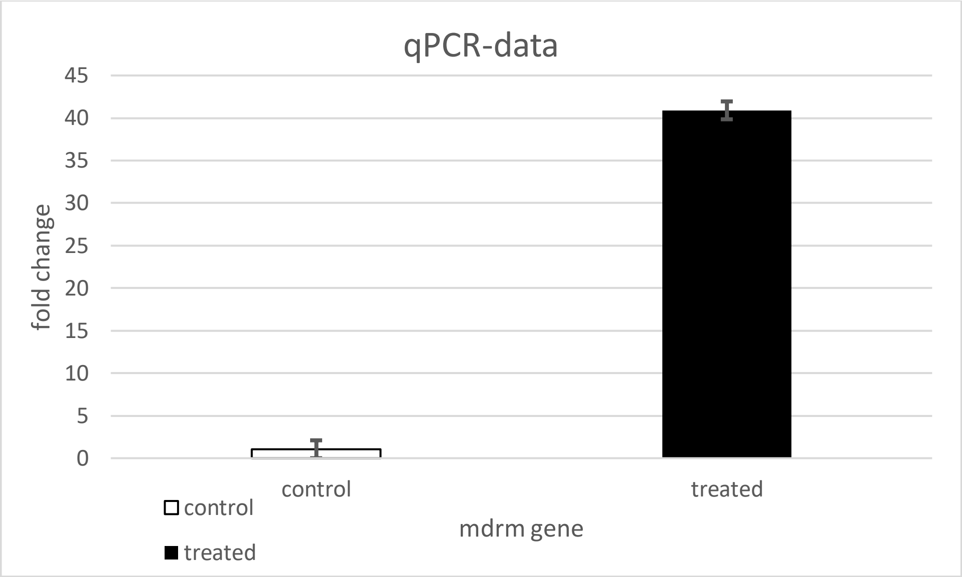

Figure 2. Mean of fold change in gene expression of mdrM gene in L. monocytogenes in compare to control one.

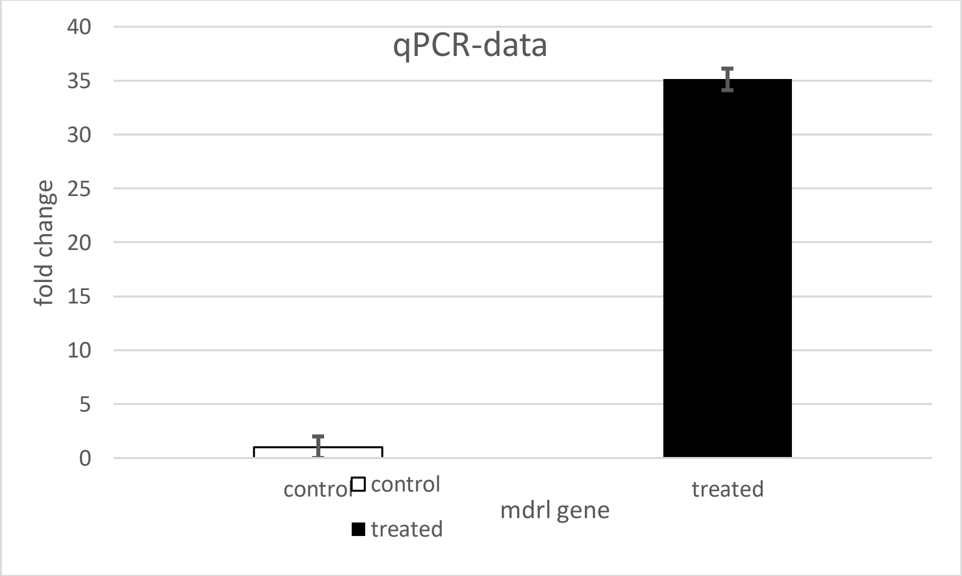

Figure 3. Mean of fold change in gene expression of mdrL gene in L. monocytogenes in compare to control one

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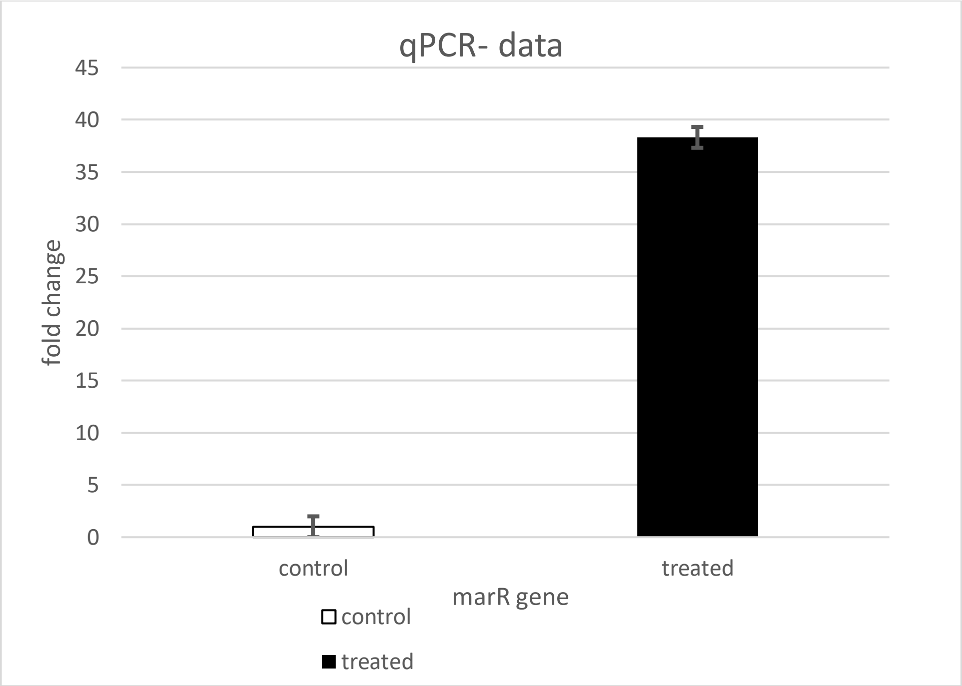

Figure 4. Mean of fold change in gene expression of mdrR gene in L. monocytogenes in compare to control one

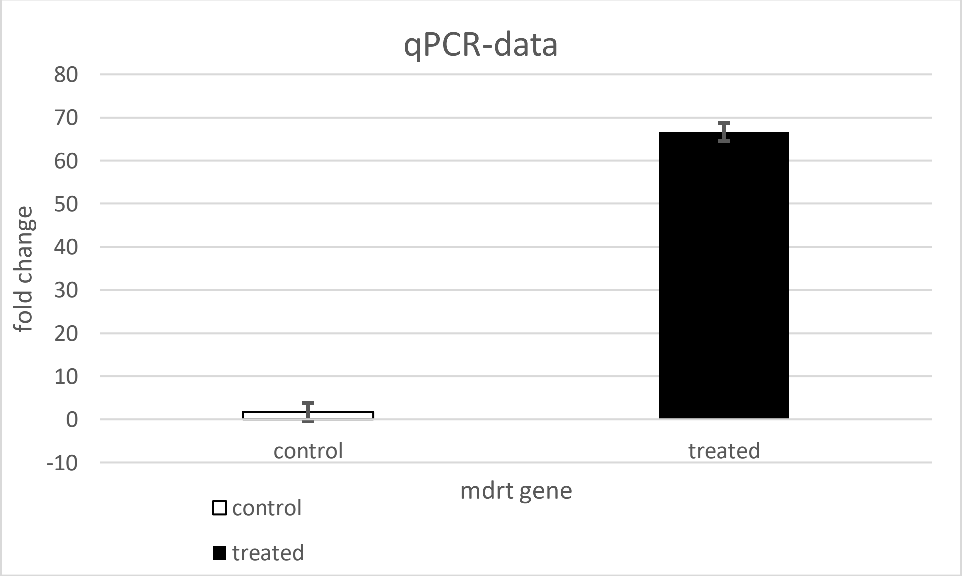

Figure 5. Mean of fold change in gene expression of mdrT gene in L. monocytogenes in compare to control one

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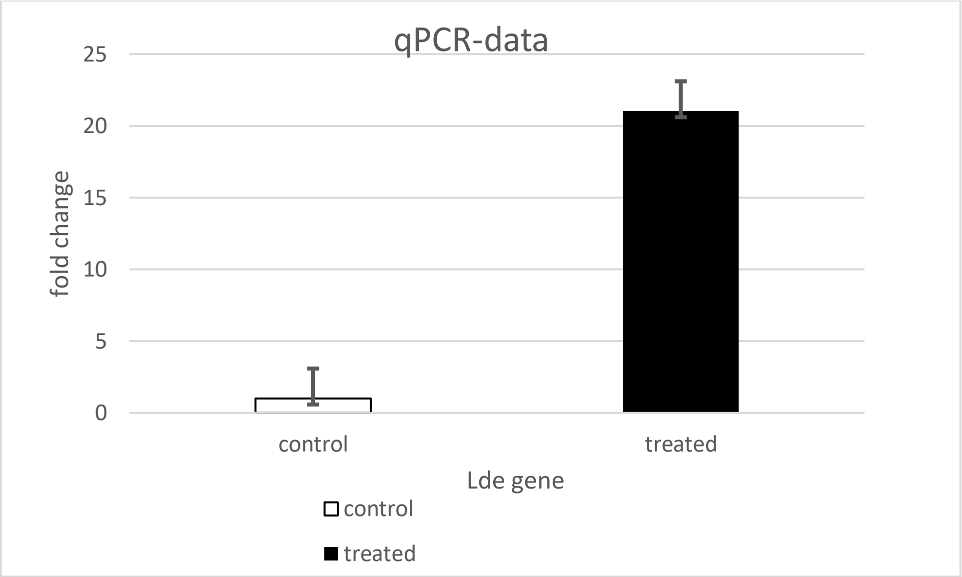

Figure 6. Mean of fold change in gene expression of lde gene in L. monocytogenes in compare to control one

According to the results above (fig. 2, 3, 4, 5 and 6), each efflux pump gene was inducible and there was increased expression after being injected into rabbit intestines, grown and then re-isolated again. The increase in expression in efflux pump genes may be related to some causes or factors, one of which is the secretion of bile into the intestine.

Also, MDR transporters (efflux pump genes) are ability to confer resistance to a wide variety of toxic compounds and drugs, including antibiotics, by utilizing proton motive force to actively extrude these compounds outside the cell [30].

In order to survive and initiate infection, enteric pathogens must resist the physiochemical defence factors in the human intestinal tract. One of these defence factors is bile, a potent antibacterial like compound in the intestine. Efflux pumps are the important mechanism by which bacteria resist antibacterial agents such as bile. Efflux of antimicrobial substances outside the bacterial cell is considered as a key factor for intestinal colonization and virulence of enteric pathogens [31]

Mammalian bile has potent antimicrobial activity, yet bacterial pathogens of the gastrointestinal tract and hepatobiliary system nonetheless persist and replicate within bile-rich environments [32].

Listeria monocytogenes colonize in the human & animal gastrointestinal tract and they have a multiple mechanism to resist the innate defences in the gut and antibacterial activity of bile. However, bile resistance in these bacteria is not fully understood [31]. Also, L. monocytogenes, encounters bile at three stages throughout its infectious cycle in vivo: in the gut during initial infection [32].

Bacterial pathogens of the gastrointestinal tract and the hepatobiliary system must be able to survive in these bile-rich environments in order to colonize host cells and disseminate during infection [33].

Therefore, bile is a stress factor that stimulates bacteria to activate virulence genes for protection and survival. Efflux pumps are one of the most important virulence genes in Listeria, which is activated

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after exposure to harmful substances to protect the bacterial cell and to remove these substances outside the cell by the mechanism of efflux pumps.

So the results above (upregulation of expression of efflux pump genes) support some evidence that suggests that L. monocytogenes have the ability to active transport bile.

Bile efflux is one of the important mechanisms, whereby bacteria resist the detergent action of bile by pumping out the bile salt from their cytoplasm to the external environment. Pumping out of antibacterial agents including bile salt needs particular proteins known as efflux pumps [34].

The specific mechanism by which listeria and enteric pathogen resists bile is not fully understood, however, accumulating evidence suggests that efflux pump plays a key role in bile resistance in a wide variety of enteric pathogens. These pumps transport bile outside of the bacterial cell and then decrease its harm.64,65,37

Some studies pointed that L. monocytogenes bile-induced transcriptome, and finding that many critical virulence factors are regulated by bile [32,35].

The multidrug efflux pumps MdrM and MdrT, previously shown to be critical for the bacterial provocation of a pathogenesis-promoting host innate immune response, are robustly and specifically induced by the bile component cholic acid. This induction is mediated by BrtA, the first identified L. monocytogenes sensor of bile, which loses the ability to bind to and repress the mdrT promoter in the presence of cholic acid [32].

Exposure of Listeria monocytogenes to 1% porcine bile results in the induction of multidrug resistance efflux pump of the major facilitator superfamily MdrM and MdrT. L. monocytogenes MdrT mutant were found more susceptible to cholic acid and bile than wild type.61

MdrT can export cholic acid, and that mdrT bacteria are significantly attenuated when exposed to cholic acid or bile.

In addition, the expression of the MdrT pump is under the negative control of the TetR-like repressor BtrA [36]. BtrA loses the ability to bind to and repress the mdrT promoter in the presence of cholic acid, thus facilitating bacterial survival in host bile-rich environments [37].

Upregulation of gene coding efflux pump proteins upon exposure listeria to a sublethal dose of bile, and increasing sensitivity to bile in pathogen lack to efflux pump (mutant) are the main methods were used in studies of bile tolerance. These studies suggest that grampositive pathogens have the ability to active transport of bile using efflux pump mechanism under genetic control [31].

Some articles documented that Bacillus cereus has the ability to germinate and sporulate in the mice gut, however, little data is available about the ability of the bacterium to survive and colonize the human gut. Transcriptional response to bile shows that induction of EmrB/QacA transporter family in the presence of bile refers to the role of this type of efflux pump in bile response [38]. A few experimental data are available about the role of Clostridium efflux pump in bile resistance.

Most of the bacteria colonize in the human & animal gastrointestinal tract have multiple mechanisms to resist the innate defences in the gut in addition to the antibacterial activity of bile.

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So, other possible reason to induce expression of mdr genes is interferon induction during listeria infection [39]. Report that it is not MdrM alone but a cohort of MDR transporters that together contribute to type I interferon induction during infection. Specifically, overexpression in listeria of two closely related MDR transporters, MdrM and MdrT, was found to trigger enhanced induction of IFN-_ by infected macrophages. However, only deletion of the mdrM gene resulted in reduced levels of IFN-secreted by infected macrophages [40]. This observation indicated that MdrM plays an active role during bacterial cytosolic growth that leads to the induction of the type I interferon response.

MdrM and MdrT were shown to be transcriptionally induced upon bacterial exposure to rhodamine 6G (R6G) and tetraphenylphosphonium (TPP), both well-known substrates of MDRs, and to confer resistance to cholic acid [16].

The function of Mdrt in L. monocytogenes is the export of cholic acid and c-di-AMP while Mdrm Export of c-di-AMP.

Conclusions

The results indicated that listeria monocytogenes efflux pump genes have a critical role in the resistance and survival of this pathogen to human bile and other antibacterial components. The efflux pumps reside in their ability to extrude a wide range of antibiotics, resulting in the emergence of multidrug resistance in many bacteria. Besides antibiotics, efflux pumps can also extrude a large variety of compounds.

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Conflict of interests

There are non-conflicts of interest.

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