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TRIAL FOR RESUSCITATION OF VIABLE BUT NON-CULTURABLE (VBNC) *L. MONOCYTOGENES* DUE TO THE EFFECT OF CHLORINE AND MAGNESIUM CHLORIDE (MGCL2) ON FOOD CONTACT SURFACE

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ABSTRACT

Many bacterial species including *L. monocytogens* have been found to exist in a viable but non-culturable (VBNC) state as a result of different stresses. VBNC cells are characterized by a loss of culturability on routine isolation by using the reference methods, which weakened their detection by conventional plate count techniques. This leads to an underestimation of total viable cells in environmental or food samples, and thus poses a high-risk level to public health. In this paper, recent findings on the VBNC state of human bacterial pathogens particularly *L. monocytogens* were approached. The viable cells after 30 min. of chlorine application (50, 100 and 200 ppm) were decreased by 100% [from 7 $\log_{10}$cfu/ml to <1 cfu/ml (0.0%)] while after 48 of resuscitation using modified detection method, the VBNC cells in case of used 50 ppm chlorine were recorded **5** $\log_{10}$cfu/ml **(**71.43%) and 2 $\log_{10}$cfu/ml dead cells (28.57%), however by using 100 ppm chlorine, VBNC cells were recorded 2.9 $\log_{10}$cfu/ml (41.43%) and 4.1 $\log_{10}$cfu/ml dead cells (58.57%) while by using 200 ppm chlorine, VBNC cells were recorded 2.8 $\log_{10}$cfu/ml (40.00%) while dead cells reached 4.2 $\log_{10}$cfu/ml (60.00%). Furthermore, by using $MgCl_2$ (0.5 and 1.0 Mol), viable cells were recorded 95.71 and 85.71 and VBNC cells (4.29 and 14.29), respectively while dead cells recorded (0.0%) in both $MgCl_2$ concentrations. Treatment with chlorine was more potent in induction of VBNC state and dead cells rather than $MgCl_2$. PCR was able to detect *inlA, prfA* and *hlyA* virulence gens and the organism had kept the virulence genes active during VBNC state, which was revealed its existence after resuscitation. The results of the present study indicated that in all cases of treatments, whether by using chlorine or $MgCl_2$ does not have the ability to kill *Listeria monocytogens*, in addition, PCR is considered a rapid method in detecting the organism and its virulence genes, while negatively samples by using PCR should be subjected to modified traditional methods of analysis. The potential influences of VBNC *L. monocytogens* on human health were discussed.

KEYWORDS: VBNC, stress, resuscitation, virulence gens, PCR, human pathogens, *L. monocytogens*, chlorine, $MgCl_2$.

INTRODUCTION

Viable but nonculturable (VBNC) bacterial cells were recognized 30 years ago which characterized by a loss of culturability on routine agar, which impairs their detection by using conventional plate count techniques. This leads to an underestimated level of total viable cells in examined samples and thus poses a risk to the public health. Since its description, a great interest was arising due to the environmental significance of this state and almost considered an adaptive strategy of bacteria to accommodate with stress conditions. In order to solve the molecular mechanisms of VBNC state creation and resuscitation, researchers in the field must be aware and overcome of common issues related to the characteristic features of VBNC cells, the hazards behind the VBNC state's induction, the models proposed for their resuscitation and the current methods to prove not only that cells are in VBNC state but also that they are able to resuscitate (**Pinto *et al.*, 2015).**

Many bacterial species have been found to exist in a viable but non-culturable (VBNC) state. The characteristics of VBNC cells, including the similarities and differences to viable, culturable cells and dead cells. Exposure to various stresses can induce the VBNC state, and VBNC cells may be resuscitated back to culturable viable cells under suitable motivators. The conditions that induce the VBNC state and resuscitation and the mechanisms underlying these two processes as well as the significance of VBNC cells and their potential influence on human health should be studied carefully **(Li *et al.*, 2014)**.

The ability to enter the VBNC state may be helpful to the bacteria, but at the same time it will represent a risk to

human health. If VBNC cells are induced, the total number of viable bacteria in the analyzed sample will be underestimated by the CFU count methods, meaning it does not represent the actual number of bacteria due to the neglection of VBNC cells. Even worse, if all bacteria in the sample are in VBNC state, the sample may be regarded as germ-free due to non-detection. For bacterial species causing human infections, the underestimation or non-detection of viable cells in quality control samples from the food industry and water distribution systems, or clinical samples may pose serious risks to the public. The risks emerge from the fact that pathogenic bacteria can be avirulent in the VBNC state but regain its virulence after resuscitation into culturable suitable media or under suitable conditions (**Du *et al.*, 2007).** This property of VBNC cells may lead to delayed and subsequently, to the recurrence of disease in patients who were thought to be cured (**Rivers and Steck, 2001**). Therefore, it is of the most importance to understand what species of human pathogens can enter the VBNC state and apply reliable detection methods to quantify the accurate population of viable cells including both culturable and VBNC cells. The gene expression of VBNC cells using RT-PCR considered the second method of detection due to the short half-life of mRNA, a positive signal indicates the presence of mRNA and thus presence of viable cells that carry out transcription. (**Gunasekera *et al.*, 2002 and Adams *et al.*, 2003).**

The VBNC state is a unique survival planning concerned by many bacteria in response to adverse environmental conditions. However, the VBNC state is a major problem when it includes pathogens, where they can bypass culture-based standard detection methods and tolerate higher disinfectant and several antimicrobial concentrations. It is therefore essential to understand the characteristics of cells in the VBNC state, which unluckily has been very difficult to be investigated experimentally until now **(Ramamurthy *et al.*, 2014).**

L. monocytogens in a VBNC state cannot cause infection (**Cappelier *et al.*, 2005; Lindback *et al.*, 2010**). Some VBNC cells are still virulent and even cause fatal infections, which may be due to rapid resuscitation in suitable environment (**Baffone *et al.*, 2003 and Cronquist *et al.*, 2012).** Moreover, **Cappelier *et al.* (2005), Wood *et al.* (2013)** and **Ducret *et al.* (2014)** suggesting that this is an adaptive strategy for long-term survival of bacteria under unfavorable environmental conditions.

Viable but non-culturable (VBNC) bacteria have been described as an organism that fail to grow and develop colonies on routine bacteriological media, but which are to be considered alive because of their capability of metabolic activity **(Oliver, 2000**). Many other pathogens including *Salmonella typhi* (**Cho and Kim, 1999)** and *Campylobacter jejuni* **(Tholozan *et al.*, 1999)** have been described to enter the state of VBNC after exposure to different stress factors such as starvation, shifts in temperature, high NaCl concentration and visible light (**Oliver, 2006).**

VBNC cells have a molecular, cellular morphology, gene expression and virulence potential difference as compared with viable culturable cells (**Li *et al.*, 2014**).

The goal of the present study was using PCR for Detecting the ability of chlorine and magnesium chloride ($MgCl_2$) of different concentrations (50, 100 and 200 ppm) to kill viable culturable cells *of L. monocytogens* experimentally contaminated the food contact surface, or converted into VBNC state as well as the developed traditional methods to resuscitate the viability of *L. monocytogens* to grow again and retained back its virulence.

Despite their non-culturability on normally tolerant media, VBNC cells are not regarded as dead cells because of various differences including 1^{st,} dead cells have a damaged membrane that is unable to retain chromosomic and plasmidic DNA, while VBNC cells have an intact membrane containing undamaged genetic information (**Cook and Bolster, 2007 and Oliver, 2010) and the 2nd** is dead cells are metabolically inactive and do not express genes, while VBNC cells are metabolically active and continue transcription and therefore, mRNA production (**Lleò *et al.*, 2000; Besnard *et al.*, 2002).**

MATERIALS AND METHODS

Preparation of Bacterial Strains and Culture Conditions

This study was accomplished by using field strain of *Listeria monocytogens*. This field strain is one of the collected bacterial strains isolated at the Reference Laboratory for Food Safety – Animal Health Research Institute – Agriculture Research Center (AHRI - ARC, Giza), Overnight cultures were prepared by selecting a single colony of the respective strains and growing into a 9 ml of fresh brain heart infusion (BHI) broth supplemented with yeast extract and incubated at 37◦C for 24h.

Induction and Confirmation of the VBNC State according to Robben *et al.* (2018). One mL. of the aforementioned bacterial suspension was centrifuged for 5 min at 8,000 ×g. Discard the supernatant and the sediment were resuspended in 1 mL of 50 ppm chlorine directly applicated in a stainless-steel tray as a food contact surface which is similar to that used in food processing factories and incubated for 1 h at room temperature. The previous procedure was repeated using the other concentrations of the respective used treatments **(Table A).**

Table (A): Antimicrobials with different concentration used for induction of VBNC condition.

Antimicrobial used	Concentration
Chlorine (Cl_2)	50 ppm
Chlorine (Cl_2)	100 ppm
Chlorine (Cl_2)	200 ppm
Magnesium chloride ($MgCl_2$)	0.5 Mol
Magnesium chloride ($MgCl_2$)	1.0 Mol

After incubation, the bacterial suspension with each antimicrobial was centrifuged for 5 min at 8,000 ×g and the sediment washed with 1 ml of 0.85% phosphate buffer saline (PBS). Cells were resuspended in 1ml BHI broth and maintained at their respective incubation temperature (37 ^{O}C).

Confirmation the presence of VBNC *L. monocytogens*
After 28 h. incubation, 0.1 ml of BHI broth was cultured onto two plates of tryptone soya agar (TSA) and Oxford agar, incubated plates overnight at 37 ^{O}C. and count positive plates.

Using Polymerase chain reaction (PCR)
To discover the presence of viable cells of *L. monocytogenes* in the BHI broth culture which give no growth on TSA agar, sample showed viable *L. monocytogens* using PCR and at the same time was negatively on TSA was resuscitated using BHI broth for another 24 hrs., followed by inoculation onto TSA agar.

Detection of virulence genes using RT-PCR
DNA extraction
DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 20 µl of proteinase K and 200 µl of lysis buffer at 56^{O}C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

Oligonucleotide Primer
Primers used were supplied from **Metabion (Germany)** are listed in table (A).

PCR amplification
Primers were utilized in a 25- µl reaction containing 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an Applied biosystem 2720 thermal cycler.

Analysis of the PCR Products
The products of PCR were separated by electrophoresis on 1% agarose gel (AppliChem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20 µl of the products was loaded in each gel slot. Gelpilot 100 bp and 100 bp plus Ladders (Qiagen, Germany, GmbH) and gene ruler 100 bp ladder (Fermentas, Germany) were used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

Table (B): Primers sequences, target genes, amplicon sizes and cycling conditions.

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
hlyA	GCA-TCT-GCA-TTC-AAT-AAA-GA TGT-CAC-TGC-ATC-TCC-GTG-GT	174	94˚C 5 min.	94˚C 30 sec.	50˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	Deneer and Boychuk, 1991
prfA	TCT-CCG-AGC-AAC-CTC-GGA-ACC TGG-ATT-GAC-AAA-ATG-GAA-CA	1052	94˚C 5 min.	94˚C 30 sec.	50˚C 40 sec.	72˚C 1 min.	72˚C 10 min.	Dickinson *et al.*, 1995
inlA	ACG AGT AAC GGG ACA AAT GC CCC GAC AGT GGT GCT AGA TT	800	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 50 sec.	72˚C 10 min.	Liu *et al.*, 2007

Statistical analysis
Statistical analyses were run in triplicate and results were reported as mean values ± standard deviation (SD). Data were subjected to analysis of variance (one-way ANOVA Excel 5.0). A p-value less than 0.05 ($p \leq 0.05$) was considered statistically significant.

RESULTS

Prevalence of viable, VBNC and dead cells of *L. monocytogens* after decontamination using different concentrations of chlorine

Growth of initial culturable cells of *L. monocytogens* (7 log_{10}cfu/ml) were inhibited completely (<1 log_{10}cfu/ml) as the date not recorded viable cells after 30 minutes of application of chlorine (50 ppm, 100 and 200 ppm) while, after Resuscitation for 48 hours on BHI, the VBNC cells were recorded 5(71.43%), 2.9(41.43%) and 2.8(40%) log_{10}cfu/ml while the dead cells were recorded 2(28.57%), 4.1(58.57%) and 4.2 (60%) using the aforementioned chlorine concentrations, respectively. From the obtained results, it could be concluded that there was significance difference (P<0.05) between all treatments as the high concentration of chlorine (200 ppm) resulted in higher dead and less VBNC *L. monocytogens* cells. This effect was less pronounced by using 100 ppm chlorine While, the concentration of 50 ppm had the least effect of the three concentrations used. The well-established concept, from the obtained results summarized that as the chlorine concentration increased, the VBNC cells of *L. monocytogens* were decreased and the dead cells were increased (Table and Fig. 1).

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Table (1): Prevalence of viable, VBNC and dead cells of *L. monocytogens* after chlorine application and resuscitation in BHI using culturable cells of 7 log10cfu/ml.

Type and concentration of antimicrobial	Initial culturable cells	Viable cells after 30 min. of application		Prevalence of VBNC After 48 hours on BHI		Dead cells	
Chlorine	7 log_{10}cfu/ml	Count	%	Count	%	Count	%
50 ppm		<1	0.0	5[a]	71.43	2[a]	28.57
100 ppm		<1	0.0	2.9[a]	41.43	4.1[a]	58.57
200 ppm		<1	0.0	2.8[a]	40.00	4.2[a]	60.00

The mean difference was significant at P<0.05 level between all treatments.

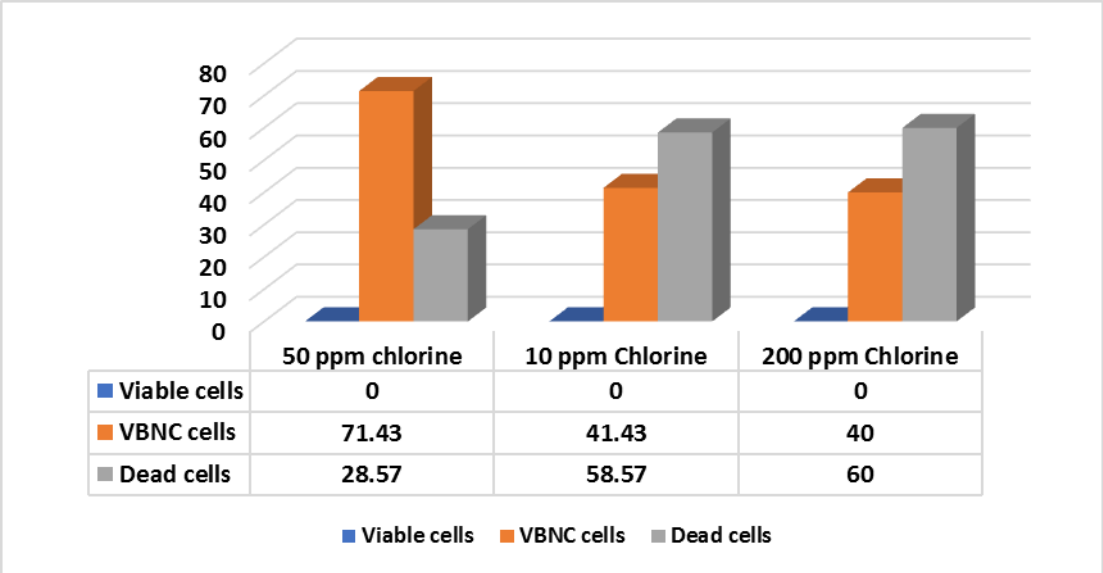

Fig. (1): Effect of different chlorine concentrations on culturable *L. monocytogens*.

Prevalence of viable, VBNC and dead cells of *L. monocytogens* after decontamination using different concentrations of $MgCl_2$

Both used concentrations of $MgCl_2$ could not eliminate *L. monocytogens* completely as the viable cells recorded 6.7(95.71%) and 6 (85.71%) log_{10}cfu/ml after 30 minutes of application of 0.5 Mol. And 1.0 Mol. Of $MgCl_2$, respectively. After 48 hos. Resuscitation on BHI for 48 hos, the count was increased, reached the same as the initial culturable cells (7 log_{10}cfu/ml) meaning that VBNC cells was recorded 0.3(4.29%) and 1.0(14.29%) log_{10}cfu/ml and dead cells recorded <1 log_{10}cfu/ml (0.0%). This data showed that as the $MgCl_2$ increased, the number of viable cells were decreased and VBNC cells were increased and these results could be clear from the presence of significance difference between mean counts of the same concentration (Table and Fig. 2). It could also be concluded that chlorine even with low concentration (1%) was more effective in induction of VBVNC state and dead cells as compared with $MgCl_2$.

Table (2): Prevalence of viable, VBNC and dead cells of *L. monocytogens* after $MgCl_2$ application and resuscitation in BHI using culturable cells of 7 log_{10}cfu/ml.

Type and concentration of antimicrobial	Viable cells after 30 min. of application		After 48 hours on BHI		VBNC		Dead cells	
	Count	%	Count	%	Count	%	Count	%
$\underline{MgCl_2}$								
0.5 Mol.	6.7^{A}	95.71	7^{a}	100	0.3^{a}	4.29	<1	0.0
1.0 Mol	6^{B}	85.71	7^{b}	100	1.0^{b}	14.29	<1	0.0

The mean difference was significant at P<0.05 level between the same capital and small letters in the same row.

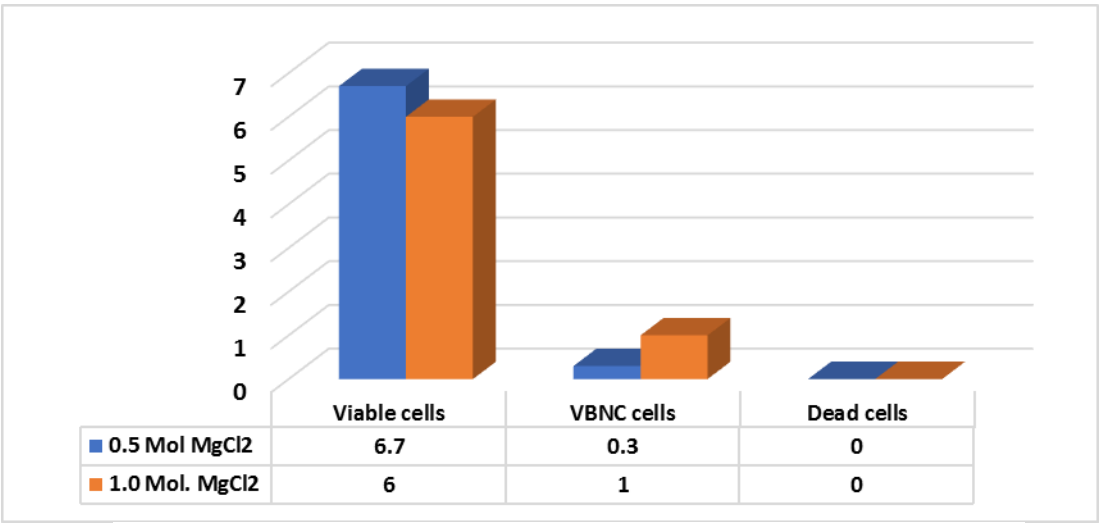

Fig. (2): Effect of different $MgCl_2$ concentrations on culturable *L. monocytogens.*

RT-PCR revealed the existence of *inlA*, *prfA* and *hlyA* genes in the field strain of *L. monocytogens* used to fulfil the experimental work. Such genes appeared again after resuscitation of VBNC cells at 800 bp, 1052 bp and at 174 bp, as shown in photography's 1,2 and 3, respectively (Table 3).

Table (3): RT-PCR detection of Virulence genes of experimentally inoculated field strain of *L. monocytogens.*

Field strain	Virulence genes before and after resuscitation		
	inlA	*prfA*	*hlyA*
L. monocytogenes	+	+	+

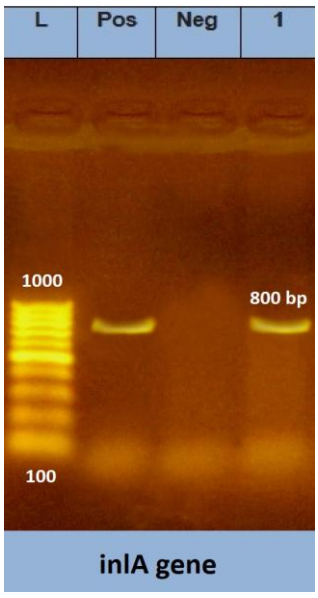

Photo (1): *inlA* gene at 800 bp

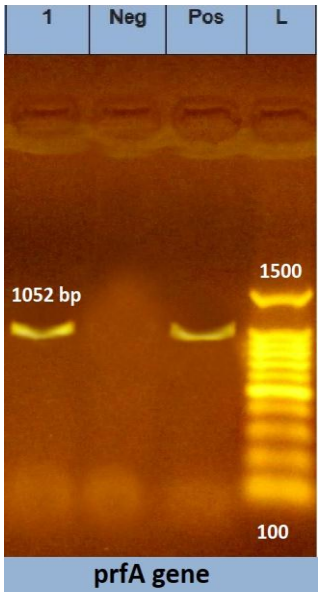

Photo (2): *prfA* gene at 1052 bp

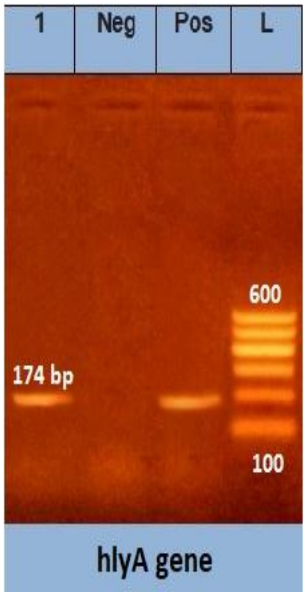

Photo (3): *hlyA* gene at 174 bp

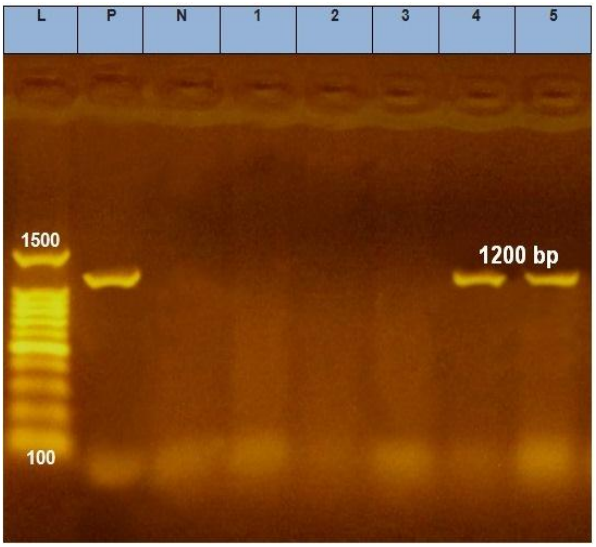

Photo (4): Agarose gel electrophoresis of RT-PCR amplified *L. monocytogens* (16S rRNA).

- Molecular Weight Marker "MWM" (100 – 1500 bp DNA ladder)
- P: Positive control L. monocytogens at 1200 bp
- N: Negative control
- Lane 1,2 and 3: Negative *L. monocytogens* in treated samples with 50, 100 and 200 ppm chlorine, respectively.
- Lane 4: Positive *L. monocytogens* at 1200 bp after treatment with 1 Mol. $MgCl_2$
- Lane 5: Positive *L. monocytogens* at 1200 bp after treatment with 0.5 Mol. $MgCl_2$

L. monocytogenes was detected after application of $MgCl_2$ of both concentrations (0.5 and 1.0 Mol $MgCl_2$) at 1200 bp while, RT-PCR could not detect the organism after chlorine application of 50, 100 and 200 ppm. Furthermore, by using modified culturable method, *L. monocytogens* grow again and retain its normal conditions and virulence factors after resuscitation in enrichment media for 48 hours (**Photo 4).**

DISCUSSION

The obtained results regarding treatment with chlorine of different concentration were agreed with **Highmore *et el.* (2018)** who stated that total culturable cells of *L. monocytogens* became VBNC after treatment with 50 and 100 ppm of chlorine, the authors added that chlorine, a sanitizer commonly used for fresh products, induces a VBNC state in the food-borne *Listeria monocytogenes*, while it was ineffective for killing total populations of the pathogens. This is proven by the current research as L. monocytogens did not eliminated completely and it was resuscitated from VBNC state into active form even after the highest concentration of added chlorine (200 ppm). Also, the results complied with **Brackett (1987**) who reported that no viable cells of L. monocytogens were detected after using 50 ppm of chlorine. Moreover, the obtained results were nearly similar to **Goncalves *et al.* (2005)** who stated that 45 mg/l (45 ppm) of chlorine resulted in decreased L. monocytogens viability to be <1 log.

Furthermore, The present data agreed with several investigators (**Goncalves and de Carvalho, 2016; Lin *et al.*, 2017; Orruno *et al.*, 2017 and Overrney *et al.*, 2017**) which stated that chlorine is an example of antimicrobial compounds that able to inducing the VBNC state in a few minutes due to its toxic stress which force the bacteria into the VBNC state, in which the bacteria appeared to loss its ability to grow through routine analysis, while in fact, they are able to resist a wide range of harmful conditions and retain its virulence and can potentially revert to the active state.

Many species have the strength to resuscitate from the VBNC state back to the culturable state when the stress is removed (**Roszak *et al.*, 1984 and Bates** and **Oliver, 2004**). This evidence supports the hypothesis that the VBNC state is a static form of life allowing the organism to wait for suitable conditions to refresh (**Rowan, 2004; Lleo *et al.*, 2007b** and **Stewart, 2012**). Furthermore, the obtained results in the present study confirmed that VBNC cells required to be cultivated on non-selective media where they complied with the results obtained by **Pinto *et al.* (2013 & 2015).** The obtained results were incompatible with **Besnard *et al.* (2000 and 2002)** who found that 2(50%) out of 4 culture collection of *L. monocytogens* strains entered the VBNC state after exposure to starvation stress, use of chemicals and exposure to high NaCl concentrations. Also, the authors added that VBNC bacteria could not be successfully resuscitated from the induced VBNC state even with addition of specific additives or changes to growth media formulations. The difference of results may be attributed to the use of field isolated instead of lab. reference strains.

$MgCl_2$ act as a stress enhancer of viable cells of *L. monocytogens*. Whereas $MgCl_2$ does not affect bacterial viability at near-neutral pH. The magnesium salt

dramatically increased the acidity to a level that had antimicrobial effect in the presence of anionic bases such as phosphate, lactate, or acetate, The antimicrobial activity of $MgCl_2$ was much stronger than that of NaCl, KCl, or $CaCl_2$ as a surface disinfectant, this observation might support the commonly stated therapeutic properties of $MgCl_2$ for the treatment of skin diseases and could contribute to understanding why salt from the Dead Sea, where Mg_2+ and Cl- are the most abundant cation/anion, has healing properties in a microbiological context **(Alarcon *et al.,* 2014).** In this context, **Ding *et al.* (2016)** stated that chemicals and preservatives are able to induce VBNC state of *L. monocytogens*. With regard to VBNC bacteria**, Oliver (2010)** stated that some of bacterial cells are still virulent and even cause fatal infections, which may be due to rapid resuscitation into highly nutritional culture media. The risks emerge from the fact that pathogenic bacteria can be avirulent in the VBNC state but regain virulence after resuscitation process under suitable conditions. This agreed with the results in the present study**.** Even without resuscitation, pathogens in the VBNC state can still be a potential threat to public health due to production of specific virulence factors or toxins **(Yaron and Matthews, 2002).**

In the present study, VBNC state was successfully induced rapidly almost 30 min after mixing of the chlorine with the culture of *L. monocytogens*. Furthermore, chlorine was more effective as compared with $MgCl_2$. This result matched those of **Zeng *et al.* (2013)** and **Weigel *et al. (*2017)** who concluded that chlorine treatment, have been shown to reduce culturability and stimulate VBNC induction rapidly**.** In this regard**, Cunningham *et al.*, 2009; Quiros *et al.*, 2009 and Robben *et al.* (2018)** stated that bacterial stress factors present in food-processing environments, such as starvation, Fermentation, chlorination, temperature, pH or using of food preservatives resulted in inducing the VBNC state in several human pathogens. In this field, **Ayrapetyan *et al.* (2015)** and **Zhao *et al.* (2017**) stated that in most industrial and food-processing environments, induction of the VBNC state and complete loss of culturability considered of a long-term process, which might take several months to occur under stable conditions.

In the present research, Resuscitation of *L. monocytogens* cells were implemented by enrichment using BHI followed by cultivation on both Oxford agar and Tryptic soy agar (TSA) and this comply with **Besnard *et al.* (2002)** and **Rowan (2004),** while **Lindback *et al.* (2010)** concluded that non of the tested VBNC cells of *L. monocytogens* regained its ability to grow and form colonies even by using BHI medium. These variations in results may be due to the type of the strain used, whether it is a reference strain or field strain and also the cause of the VBNC state either by starvation, temperature or use of chemicals which differ in their mode of action and accordingly differ in their effect.

Tamburro *et al.* (2015) stated that virulence of *Listeria monocytogenes* depends on the activity of well-characterized virulence factors including *inlA*, *hlyA* and *prfA* genes. Moreover, **Kazmierczak *et al. (*2006)** stated that *PrfA* appears to be more important as a virulence gene during the systemic and intracellular stages of infection. In this respect, **Lindback *et al.* (2010)** studied the viability and potential virulence of VBNC *L. monocytogenes* strains through transcription of mRNA by using RT-PCR and he mentioned that Listeriolysin O (*hly*) is an important virulence factor in *L. monocytogenes*, expression of *hly* in the VBNC state will indicate a virulence potential present in the bacteria. Transcripts of *hly* were detected in VBNC cells of all the 16 strains by RT-PCR.

The obtained results showed the failure of the RT-PCR to detect VBNC cells of *L. monocytogens* 30 minutes post treatment with chlorine, while the organism was present in a VBNC state and was activated and attained its virulence genes by the cultivation method. These results were compatible with **Bernstein *et al.* (2002), Pease *et al.* (2002)** and **Rieu *et al.* (2007)** as they concluded that RT-PCR is not a quantitative method and may not be ideal for detection of mRNA half-life of VBNC *L. monocytogens* in culturable cells.

4- CONCLUSION AND RECOMMENDATIONS

A- *Listeria monocytogenes* can enter into the VBNC state is of interest for the food industry since routine bacteriological testing will fail to detect non-culturable organisms in the processing environment and in the products.

B- The VBNC state of *L. monocytogens* can be induced almost instantly if they are exposed to strong bactericidal (Cl_2) and for less extent to ionic compounds (MgCl2), conditions which are widespread throughout industrial, commercial, municipal and domestic environments.

C- In all circumstances, cleaning and disinfection strategies currently applied to culturable bacteria found to be not sufficient as a proper intervention against VBNC pathogens as the VBNC state of food-borne pathogens still pose a potential public threat, especially as bacteria in the VBNC state are invisible for growth-based microbiological methods, which as a result may underestimate pathogen risk in routine diagnostics.

D- Furthermore, research is necessary to determine the VBNC resistance and tolerance as well as the levels of this risk and the possible consequences of VBNC pathogens on public health.

E- Viable but non-culturable (VBNC) bacteria have significant implications for microbiologists in all fields, especially in the context of detection, interference and chemotherapy when challenge with VBNC pathogens. Also, the VBNC status approached is suitable for high-throughput susceptibility screening of bacterial cells in the VBNC state.

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