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Detection and Control of Viable but Non-Culture *Escherichia coli* Using Some Selective Sanitizers

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Abstract | Many pathogens, including *Escherichia coli* (*E. coli*), can enter a viable but non-culturable (VBNC) state in response to environmental stress. In this state, bacteria cannot grow on standard media but retain certain features of viable cells, such as cellular integrity, metabolic activity, and virulence. Therefore, this study examined the effect of chlorine and hydrogen peroxide as common chemical sanitizers on inducing *E. coli O157* into a VBNC state. The results showed that after 30 minutes of treatment with 200, 100, or 50 ppm chlorine, the numbers of culturable *E. coli* significance P<0.05 dropped (100%) from 6.93±0.04 $\log_{10}$ CFU/mL to less than 1 CFU/mL (0.0%). However, after 48 hours of resuscitation using sodium pyruvate as nourished media, the VBNC cells in case of used 200, 100 and 50 ppm chlorine were recorded 3.13±0.04, 4.08±0.04, and 4.49±0.02 CFU/mL, respectively. In contrast, application of 0.1, 0.3-, and 3-mM concentrations of hydrogen peroxide for 30 minutes could not induce *E. coli o157* into VBNC state but only significancy (P<0.05) decreased the count of viable cells from 6.93±0.04 (100%) to 6.49±0.03 CFU/ml (93.7%),6.04±0.06 CFU/ml (87.2%), and 4.79±0.01 CFU/ml (69.1%), respectively. Thus, treatment with chlorine was more potent in induction *E. coli* O157 into VBNC state after 30 min. of application rather than hydrogen peroxide which failed in inducing the bacteria into VBNC at the same time points. The PMA-qPCR method, which was used to find the rfbE gene of *E. coli* O157, showed that the VBNC cells were still alive and working. This suggests that PMA-qPCR could be a useful way to find the VBNC state. This study concluded that in all treatments, whether by using chlorine or H_2O_2 does not have the ability to kill *E. coli* O157. The resuscitation of VBNC *E. coli* cells has been studied for the purpose of risk control of recovered pathogenic bacteria Therefore, From the food safety point of view new formulation are highly required to control VBNC *E. Coli*. In addition, PAM-q PCR is considered a rapid method in detecting the VBNC organism. The potential influences of VBNC *E. coli* O157 on human health were discussed and the better ways to clean surfaces that human touch.

Keywords: *Escherichia coli O157:H7*, Chlorine, Hydrogen peroxide, PMA-qPCR, Resuscitation

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INTRODUCTION

In recent years, concerns about microbial food safety have globsally risen, accompanied by an increasing risk of severe foodborne diseases. For instance, the incidence of illnesses and deaths caused by major pathogens, including *Escherichia coli (E. coli)*, has surged worldwide (Burgess *et al*., 2016). *Escherichia coli* O157:H7 was one of the major foodborne pathogens as it causes a serious threat to public health, water, and the food and beverage industry (Furukawa *et al*., 2018). Food and surfaces contamination by *E. coli O157:H7* are associated with bacterial outbreaks in beef, dairy, juice, tomatoes, eggs, poultry, seafood, and lettuce (Chen *et al*., 2021) *E. coli* O157, known for producing Shiga toxin or Shiga-like toxin, can lead to serious conditions such as hemorrhagic colitis and hemolytic uremic syndrome in humans (Neil *et al*., 2012). Shiga toxin can cause bloody diarrhea and severe hemolytic uremic syndrome, potentially causing kidney failure. As a typical foodborne pathogen, *E. coli* O157 can colonize food systems and drinking water (Bourely *et al*., 2018). Several outbreaks of *E. coli* O157:H7 associated with food consumption have been reported by the Centre for Disease Control and Prevention (CDC); for example, in 2018, a multistate outbreak was reported in the USA linked to romaine lettuce; 210 people fell sick, 96 were hospitalized, 27 developed haemolytic uremic syndrome (HUS) (i.e., permanent renal failure) and 5 people died (CDC, 2018).

Viable but Non-Culture (VBNC) means cells are in state of dormancy, which induced because of unfavorable conditions such as an unsuitable temperature (high or low), high osmotic concentrations, low oxygen level, UV radiation, decontamination treatments as using sanitizer, change atmosphere during packaging, food preservation methods, heavy metals, and pasteurization of milk (Maertens *et al*., 2021).When study the effect of temperature as stress factor on *E. coli* O157:H7, it was found that *E.coli* cells were induced to VBNC state at two different temperatures at + 4 °C and – 20 °C (Li *et al*., 2020). VBNC (viable but non-culturable) cells exhibit specific characteristics, such as reduced cell size, which leads to cell dwarfing and rounding. These changes happen because of changes in how proteins, fatty acids, and peptidoglycans are made. These are important for making up the cell wall and membrane (Progulske-Fox *et al*., 2022). The VBNC state is a temporary condition of low metabolic activity where bacterial cells do not divide but maintain intact cell membranes, continue gene expression, and can regain viability upon resuscitation (Zhao *et al*., 2017). The VBNC state may be good to the bacteria, but at the same time it will cause risk to human health. As if bacteria induced into VBNC state, the total number of viable bacteria in the tested sample does not represent the actual CFU number of bacteria as not recorded of VBNC cells. Also, the sample may be considered germ-free if all bacteria enter VBNC state, due to non-detection by conventual methods. 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 cause serious problems to human health. The risks rise in fact that the VBNC pathogenic bacteria can regain its virulence after resuscitation into culturable suitable media or under suitable conditions (Du *et al*., 2007).

Recently, there has been growing interest in bacterial regeneration following disinfection, particularly regarding VBNC cells induced by such treatments (Wang *et al*., 2021). Bacteria in the VBNC state do not form colonies on standard detection agar plates when exposed to certain environmental stresses. Despite their inability to be detected by routine methods, VBNC cells retain their potential to cause food spoilage and cause food safety risks, making the VBNC state of *E. coli* O157 a significant concern in the food industry (Liu *et al*., 2018). Sanitizers, especially chlorine and H_2O_2 are the most popular disinfectant used, as they are needed to maintain the microbiological quality of Processed washing water and contact food surfaces for avoiding cross-contamination (Gombas *et al*., 2017). Chlorine is the most widely used sanitizer in the fresh produce industry, and the effectiveness of sanitizers is typically assessed using plate counts (López-Gálvez *et al*., 2019).

Resuscitation refers to the process by which VBNC (viable but non-culturable) bacteria regain their metabolic activity and ability to grow, which requires specific conditions. These conditions include removing stress factors, adding nutrients, stabilizing osmotic pressure, and degrading hydrogen peroxide. This phenomenon has been documented in several bacterial species, such as *E. coli* O157 and *L. monocytogenes* (Dong *et al*., 2020). Several in vitro methods have been evaluated for recovery of VBN bacteria. One recovery method depends on addition of peroxide-degrading agents such as sodium pyruvate or catalase to the media, where these agents able to protect cells from oxidative stress. Recently, researchers reported that supplementation of agar medium with sodium pyruvate or catalase) regained culturability to VBNC *E. coli* O157 (Mizunoe *et al*., 1999). Sodium pyruvate was taken up by starved and cold-stressed VBNC *E. coli* cells, as they have many component systems BtsSR and YpdAB which respond to extracellular pyruvate through the high affinity pyruvate/H^+ symporter, membrane-integrated histidine kinase (BtsS/YpdA) that can perceive pyruvate and (BtsR/YpdB) as cytoplasmic response regulator mediate *btsT* expression Therefore, VBNC cells may utilize pyruvate as an alternative carbon source and correspondingly fine-tune their transport capacities and metabolism for resuscitation (Vilhena *et al*., 2019).

The concept of resuscitation is now broadly recognized as the recovery of VBNC cells, which involves restoring their metabolic activity and culturability. This recovery is typically assessed using methods such as plate counting or turbidity measurement (Yang *et al*., 2022).

The ability of VBNC (viable but non-culturable) bacteria to resuscitate depends on the duration of the VBNC state and the intensity of external stress factors. Numerous bacterial species have been observed to recover from the VBNC state under specific conditions, including *Salmonella enteritidis*, *E. coli* O157, and *L. monocytogenes*. Resuscitation of VBNC cells can be achieved through various methods, such as alleviating stress factors and providing nutrient supplementation, which makes the environmental conditions more conducive to cell growth and division (Zhao *et al*., 2020). Studying the *E. coli* O157:H7, cells induced into VBNC state at high temperature. The results indicated that Shiga toxin genes (*stx1, stx2*) and virulence genes (*eae and hlyA*) were highly expressed in VBNC cells and genes, especially *eae* and *stx2* genes were expressed at a higher level in VBNC cells than in culturable cells, so higher virulence in VBNC cells was considered which could be associated with foodborne outbreaks (Fu *et al*., 2020). Also, cytotoxicity tests were performed on Vero cells to quantitatively evaluate Stx production in induced VBNC *E. coli* O157:H7 cells induced to VBNC state by chlorinated and chloramine water and found that VBNC cells retain their ability to produce Stx under all conditions and have high levelss of Stx (Liu *et al*., 2010). So, virulence still presents in *E. coli* O157:H7 cells either VBNC state or in resuscitated cells.

Even though VBNC bacteria can't grow on normal growing media, they can be told apart from culturable cells, damaged cells, and dead cells by how well they grow, how well their membranes are intact, and how active their metabolism is. These distinctions allow for the development of various detection methods to identify and quantify VBNC bacteria (Truchado *et al*., 2020). Many bacteria capable of entering the VBNC state are pathogenic and may still produce toxins or regain their infectivity and pathogenicity upon resuscitation, potentially leading to human illness or food spoilage (Yoon *et al*., 2021). Molecular methods, such as polymerase chain reaction (PCR), are commonly used for nucleic acid amplification and can identify target strains within a few hours. However, PCR cannot differentiate between viable and dead cells, as DNA from dead cells can remain intact, resulting in false-positive results (Liu *et al*., 2014). To solve this problem, real-time quantitative PCR (qPCR), a fast way to count VBNC cells that doesn't depend on culture, is a good option because it only amplifies healthy cells (Wulsten *et al*., 2020). The principle behind PAM-qPCR-based detection is that the mRNA of dead cells becomes undetectable after a short period, while the mRNA of live cells remains, making it possible to detect all viable cells (Lee *et al*., 2021). qPCR can also be improved with certain chemicals, such as propidium monoazide (PMA), which can get into cells with permeable membranes and, when exposed to light, damages nucleic acids in a way that can't be fixed. This stops PCR from amplifying cells that aren't alive (Li *et al*., 2014).

The study's goal was to find out how well different concentrations of chlorine and hydrogen peroxide could put *E. coli O157* into the VBNC (viable but non-culturable) state after it was exposed to food-contact surfaces in an experiment. The study also aimed to confirm this state using the PMA-qPCR technique and evaluate the effectiveness of traditional methods in resuscitating *E. coli* O157 to restore its viability and virulence.

MATERIALS AND METHODS

Preparation of bacterial strains and culture conditions

E. coli O157:H7 (NCTC12241/ATCC25922) strain was obtained from the Reference Laboratory for Food Safety, Animal Health Research Institute, Agriculture Research Center (AHRI-AARC, Giza). The culture of *E. coli* was prepared by inoculation on LB broth (L3022, Sigma-Aldrich) and incubation at 37 °C for 24 hours to reach the stationary phase. For test suspensions, 1.0 mL of the liquid culture was centrifuged at 3000 rpm for 15 min at 4 °C and resuspended in 1 mL sterile 0.85% NaCl solution. The centrifugation and resuspension were repeated twice to wash off the LB nutrient medium. Finally, *E. coli* suspension had the correct inoculum level of 10^7 cfu/mL.

Sanitizers and chemicals

In this study, was obtained sodium hypochlorite from EL-Gomhoria Company in Tanta, Egypt. We adjusted solutions of sodium hypochlorite (NaClO) (>5%, Kanto Chemical) to reach concentration of 50, 100, and 200 ppm free chlorine using distilled water. The concentration of free chlorine was measured with the photometer Spectroquant NOVA 60 (Merck, Darmstadt, Germany). Chlorine treatment times and concentrations were selected based on commonly utilized conditions according to Tolba *et al*. (2020). Hydrogen peroxide was prepared from a 30% hydrogen peroxide solution from Merck at concentrations of 0.1 mM, 0.3 mM, and 3 mM according to (Munna *et al*., 2013). Sodium pyruvate, as chemical factors trigger the exit of cells from the VBNC state (Zhong *et al*., 2009). Stainless-steel trays preparation: Stainless steel type 304 with a no. 4 finish was cut into SSCs (2 by 5 cm^2),then were washed by immersion in a hot alkali detergent solution (FS Pro-Chlor, Zep, Atlanta, Ga.) at 808C with sonication for 20 min in an ultrasonic water bath (model 250D, VWR,

Chester, Pa.), rinsed in distilled water, sonicated in a 15% phosphoric acid solution at 808C for 20 min, and rinsed in distilled water with agitation for 20 min. Cleaned SSCs were transferred to a beaker and dry, finally were sterilized in an autoclave (20 min at 121 ˚C, 1 bar pressure).

VBNC State Induction, according to Robben *et al*. (2018)

One mL of *E. coli O157:H7* (10^7 cfu/mL) suspension was centrifuged for 5 min at 8,000 rpm. After that, the supernatant discarded then resuspended the sediment in 1 mL of 200 ppm chlorine (GI). The mixture directly applied to a stainless-steel tray, like those used in food processing factories, and incubated it for 30 minutes at room temperature for attachment and pH was 7.0 during the incubation period. The previous procedure was repeated using the other concentrations of the respective treatments. GII: 100 ppm chlorine, GIII: 50 ppm chlorine, GIV: 0.1 mM H_2O_2, GV: 0.3 mM H_2O_2, and GVI: 3 mM H_2O_2. The initial count of control positive was without sanitizer. *E. coli O157:H7* was mixed with each sanitizer and centrifuged it for 5 minutes at 8,000. The sediment was washed with 1 ml of 0.85% phosphate buffered saline (PBS). Cells were resuspended in 1mL of BHI broth and maintained at 37˚C.

Culturability of *E. coli O157:H7*

The plate counting method was used to determine the culturable cell number after 30 min. To sum up, 1 mL from each stainless-steel tray was diluted ten times in 9 mL of peptone water (0.1%). Then, 0.1 mL was spread out on Eosin Methylene Blue and other spread-plated on TSA agar media and left to grow at 37˚C for 48 hours to count *E. coli O157:H7* cells that had been stressed by disinfectants. When the culturable number is >1 CFU/mL, the cells are considered non-culturable and may enter the VBNC state**.** The number of VBNC cells was calculated as the difference between the numbers of viable and culturable cells.

Resuscitation of VBNC *E. coli O157:H7*

The VBNC bacteria cells to resuscitate after chlorine sanitation where culturable number is >1 CFU/mL or bacteria were not detected. The bacterial suspension with each sanitizer was centrifuged for 5 min at 8,000 rpm, at 4 ˚C. The supernatant was removed, and the sediment pellet resuspended in 1 mL of 0.85% phosphate buffered saline (PBS) at pH = 7 the resuspension cells were also added to 9 mL of Brain Heart infusion (BHI) broth supplemented with 0.1% of sodium pyruvate, and in 9 mL of Modified Buffered Peptone Water with Pyruvate (mBPWp) (Jasson *et al*., 2009). Samples were incubated for 48 h at 37 ˚C. Then, the culturability was tested using the plate counting method on EMB agar media, and the positive plates were counted. All experiments described here were performed in triplicate.

PMA-qPCR confirms the presence of VBNC *E. coli O157:H7*.

Propidiummonoazide staining (PMA)

Method described by Taskin *et al*. (2011) was used for staining. To make a stock solution of 10 mM, PMA (Sigma-Aldrich) was mixed with 20% dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO). This solution was then kept at -20˚C. The PMA stock solution was transferred to 500µl culture mixtures at a final concentration of 100µM. All manipulations of PMA solution were performed under minimal light to prevent any potential chemical change in PMA structure. Following 10 min of dark incubation, samples were exposed for 5 min to a 500-W halogen light source.

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 °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

The primers used were obtained from Metabion (Germany) and are listed in Table 1.

PCR amplification

It took 25µl of DNA template, 12.5 µl of 2x QuantiTect SYBR Green PCR Master Mix (Qiagen, Gmbh, Germany), 0.5 µl of each primer at a concentration of 20 pmol, and 8.5 µl of water to use the primers. The reaction was performed in a MX3005P real-time PCR machine (Agilent, 2012).

Table 1: Primers sequences, target genes, amplicon sizes and cycling conditions.

Target gene	Primers sequences	Primary denaturation	Amplification (40 cycles)		
			Secondary denaturation	Annealing	Extension
E. coli O157 rfbE	GTAAATATGTGGGAACATTTGG	94˚C	94˚C	60˚C	72˚C
	GGCCTTTAAAATGTAAACAACGG	5 min	30 sec	30 sec	30sec

Table 2: Prevalence of *E. coli O157:H7*: viable, VBNC and dead cells after chlorine application on food contact surfaces.

Treated chlorine groups	C +ve: Initial culturable count cells	Viable cells after 30 min of decontamination		VBNC count after 48 h in sodium pyruvate		Dead cells	
	6.93±0.04^{Aa} log	Count	%	Count	%	Count	%
GI		<1	0.0	3.13±0.04^{d}	45.2	3.80±0.05^{B}	54.8
GII		<1	0.0	4.08±0.04^{c}	58.9	2.85±0.02^{C}	41.1
GIII		<1	0.0	4.49±0.02^{b}	64.8	2.44±0.04^{D}	35.2

N.B: % was calculated according to the initial culturable cells of control (C+ve) sample 6.93±0.04 log cfu/ml. C: Control positive *E. coli*; GI: 200 ppmCL_2 + *E. coli*; GII: 100 ppm CL_2+ *E. coli*, and GIII: 50 ppm CL_2+ *E. coli*. The mean values carrying different superscript letters are significantly different at P<0.05.

Calculation of fold change

The mean 10-fold amplification under untreated samples was calculated according to the theory, which says that a 10-fold should take 3.32 cycles.

Statistical analyses

Statistical analyses were run in triplicate and results were reported as mean values and standard error (Mean±SE). Use of Statistical Packaging for Social Science (SPSS) Ver. 20. (one-way ANOVA, Excel 7.0 A p-value less than 0.05 ($p \leq 0.05$) was considered statistically significant.

RESULTS AND DISCUSSION

Effects of chlorine and H_2O_2 on viable, VBNC, and dead cells of *E. coli O157H7*

In the present study, we noticed a significant difference (P<0.05) between all used concentrations chlorine treatments. The growth of initial culturable viable *E. coli O157* (6.93 log10 cfu/mL) was completely inhibited because conventional plate counts did not detect any viable cells (<1 log10 cfu/ml) after 30 minutes of 200, 100, and 50 ppm chlorine application (Table 2 and Figure 1). On the other hand, there was a significant difference (P<0.05) between different concentrations H_2O_2 treatments. Treatment *E. coli O157* cells with 0.1 mM, 0.3 mM, and 3.0 mM hydrogen peroxide for 30 minutes only decreased the number of culturable cells from 6.93±0.04 log10cfu/mL to 6.49±0.03 (93.7%), 6.04±0.06 (89.2%), and 4.79±0.1 (69.1%) log CFU/ml, respectively (Table 3 and Figure 2).

Resuscitation of VBNC cells of *E. coli* after using different concentrations of chlorine

During the regrowth phase, the viable *E. coli* concentration showed diverse patterns depending on the concentration of chlorine treatment. After 48 hours of resuscitation with sodium pyruvate, the VBNC cells were recorded at 4.49 ± 0.02 (64.8%), 4.04 ± 0.04 (58.9%), and 3.13 ± 0.04 (45.2%) log cfu/mL, while the dead cells were recorded at 2.44 ± 0.04 (35.2%), 2.85 ± 0.02 (41.1%), and 3.8 ± 0.05 (54.8%) for chlorine (200, 100, and 50 ppm, respectively).

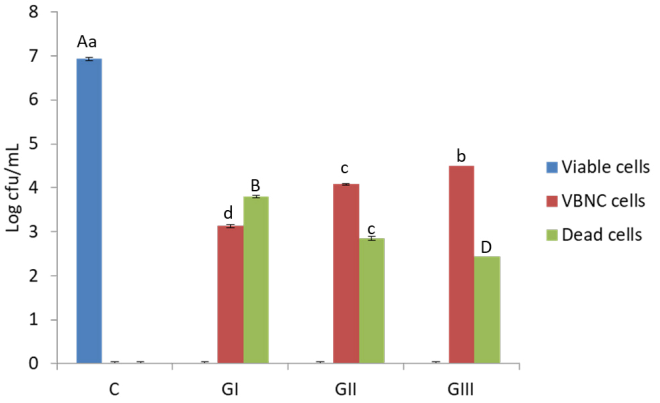

Figure 1: Effect of different chlorine concentrations on culturable *E. coli* after 30 min of decontamination. C: Control positive *E. coli*; GI: 200 ppmCL_2 + *E. coli*; GII: 100 ppm CL_2+ *E. coli*, and GIII: 50 ppm CL_2+ *E. coli*. The mean values carrying different superscript letters are significantly different at P<0.05. Experiments were performed in triplicate, and error bars represent standard error of the mean.

Table 3: Prevalence of *E. coli* O157:H7: viable, VBNC and decreased cells after H_2O_2 application on food contact surfaces.

Treated H_2O_2 groups	C: Initial culturable count cells	Viable cells after 30 min of decontamination		Count of decreased cells after 30 min of decontamination	
	6.93±0.04^{a} log cfu/ml	Count	%	Count	%
G IV		6.49±0.03^{b}	93.7	0.44±0.06^{d}	6.3
G V		6.04±0.06^{c}	87.2	0.89±0.10^{c}	12.8
G VI		4.79±0.10^{d}	69.1	2.24±0.08^{b}	32.3

N. B: % was calculated according to the initial culturable cells of control (C+ve) sample (6.93 log cfu/ml). *GIV;* H_2O_2 0.1mM+ *E. coli, GV;* H_2O_2 0.3Mm+ *E. coli* and *GVI* H_2O_2 3Mm+ *E. coli*. The mean difference was significant at P<0.05 level between all treatments.

The *rfbE* gene of *E. coli O157H7* was detected using the PMA-qPCR technique

The *rfbE* gene was present in all the groups that were tested at different cycle thresholds (CT) (Table 4 and Figure 3). The fold change was 3.96 times smaller in the GI group that was treated with 200 ppm CL2, 2.52 times smaller in

the GII group that was treated with 100 ppm CL2, and 1.77 times smaller in the GIII group that was treated with 50 ppm CL2 compared to the control group. So, these results confirm the presence of VBNC in *E. coli* O157:H7.

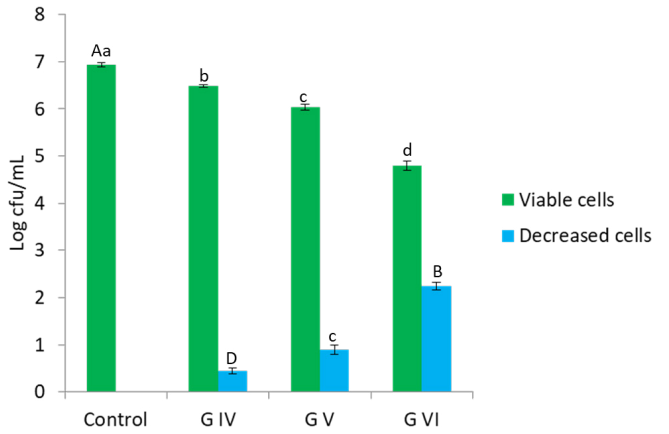

Figure 2: Effect of different H_2O_2 concentrations on culturable *E. coli* after 30 min of decontamination. Control: Control positive *E. coli*; *GIV;* H_2O_2 0.1mM+ *E. coli, GV;* H_2O_2 0.3mM+ *E. coli* and *GVI:* H_2O_2 3mM+ *E. coli*. The mean values carrying different superscript letters are significantly different at P<0.05. Experiments were performed in triplicate, and error bars represent standard error of the mean.

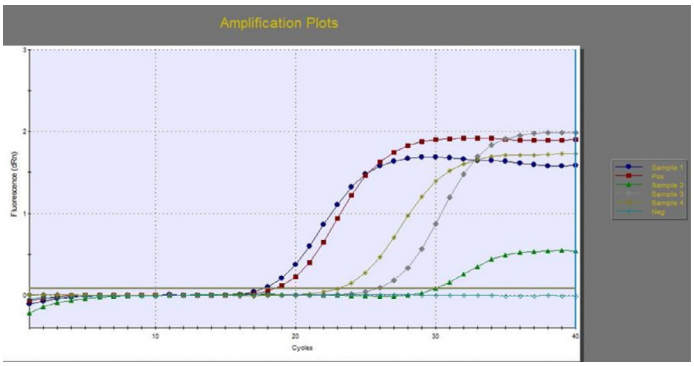

Figure 3: Detection of rfbE gene by qPCR, Sample 1: Control positive, Sample 2: 200 ppm chlorine, Sample 3: 100 ppm chlorine, Sample 4: 50 ppm chlorine.

Table 4: Result of PMA-qPCR for detection of *rfbE* gene.

Sample No	Sample ID	*E. coli O157 rfbE*		No. of fold change decreased
		Result	CT	
1(C)	*E. coli* (control +ve)	+	17.01	-
2 -(GI)	*E. coli* + 200 ppm CL	+	30.18	3.96
3- (GII)	*E. coli* + 100 ppm CL	+	25.47	2.52
4- (GIII)	*E. coli* + 50 ppm CL	+	22.91	1.77

N.B.: sample 1: C: control positive; sample 2: GI (200 ppm CL_2); sample 3: GII (100 ppmCL_2) and sample 4: GIII (50ppmCL_2).

Since its initial discovery in *Escherichia coli* in 1982, the VBNC (viable but non-culturable) state has become a well-recognized phenomenon observed in microorganisms exposed to stressful conditions. This state has been found in various environments, including water, air, soil, foods, medical facilities, food processing areas, and contact surfaces (López-Gálvez *et al.*, 2019). VBNC cells can be pathogenic and have the potential to survive until environmental conditions become favorable for their growth and division, thereby posing a significant threat to human health (Yang *et al.*, 2022).

E. coli O157 capturability and viability were assessed after 30 minutes using both cultural methods and resuscitation techniques. The difference between culturable and viable cell counts indicated the number of VBNC (viable but non-culturable) cells. In our study, various concentrations of chlorine were tested, as shown in Table 2 and Figure 1. The initial count of *E. coli* O157 was 6.93±0.04 log cfu/ml. After being treated for 30 minutes with 200 ppm, 100 ppm, and 50 ppm of chlorine for GI, GII, and GIII group, respectively, the *E. coli* count was completely stopped (<1 log10 CFU/ml), and standard plate counting did not show any viable cells. After 48 hours of resuscitation using sodium pyruvate, the VBNC counts for GI, GII, and GIII were recorded. It was found that there was a significant difference (P<0.05) between the three treatments. The treatment with the chlorine (200 ppm) had the fewest VBNC *E. coli O157* cells by 3.13±0.04cfu/ml and highest count of dead cells 3.8 cfu/ml (54.8%), followed by Chlorine at 100 ppm by 4.08±0.04cfu/ml and count of dead cells by 2.85cfu/ml (41.1%), which was more effective than 50 ppm, as had the highest count of dead cells by 4.49±0.02cfu/ml and lowest dead cells count by 2.44 cfu /ml (35.2%), respectively.

The findings show that the VBNC (viable but non-culturable) state in *E. coli O157* changes depending on the concentration at same constant time. As chlorine concentration increased, the count of VBNC *E. coli* O157 cells decreased, while the number of dead cells increased. These findings are consistent with those reported by Tolba *et al.* (2020), who observed that chlorine concentration at 200ppm,100ppm and 50ppm could induce the VBNC state in *L. monocytogenes* after 30 min and the following a changed detection of resuscitation method for 48 hours, VBNC *L. monocytogenes* cells were found to be 2.8 log10 CFU/mL (40.00%), 2.9 log10 CFU/ml (41.43%), and 5.0 log10 CFU/mL (71.43%) at 200 ppm, 100 ppm, and 50 ppm chlorine concentrations, respectively. Also, Liu *et al.* (2009) who recorded induction of *E. coli O157* into VBNC state using low concentration 0.7mg/l monochloramines and 1.5 mg/l total chloramine. Chen *et al.* (2018) reported similar result for induction of *Escherichia coli* into a VBNC state. As after using different concentrations of chlorine and chloramine 1, 2, 3, and 4 mg/L, the counts of culturable *E. coli* cells decreased from 10^6 CFU/mL to 0 CFU/mL at 120-60 -30 and 5 min post treatment.

However, viable cell counts were still approximately 10^3–10^5 cells/mL at the same time points Additionally, the current data align with findings from several researchers (Lin *et al*., 2017; Orruno *et al*., 2017) who reported that chlorine, as an antimicrobial agent, can induce the VBNC state in a matter of minutes. This occurs because the toxic stress from chlorine forces bacteria into the VBNC state, where they lose their ability to grow on routine media. Despite this, these bacteria can survive various harsh conditions, retain their virulence, and potentially revert to an active state.

Several studies have reported the varying effective concentrations of free chlorine required to prevent the induction of the VBNC (viable but non-culturable) state. Zhong and Zhao (2018) suggested that a concentration as low as 10 mg/L of free chlorine could be sufficient. In contrast, other research indicates that higher concentrations, ranging from 20 to 25 mg/L, may be necessary (Tudela *et al*., 2019). The different minimum effective concentrations of free chlorine needed to stop VBNC induction could be due to differences in how the experiments were set up, such as the size of the study (laboratory vs. pilot scale), the contact time, the presence of organic matter, and how long the residual sanitizer concentrations were kept. Furthermore, pH can have a significant impact on chlorine's antimicrobial activity (Martin *et al*., 2013).

The resuscitation of VBNC cells has been widely studied for the purpose of risk control of recovered pathogenic or spoilage bacteria and how could further applications of resuscitated VBNC bacteria in the food industry. In the present research, Resuscitation of *E. coli O157* cells were done by enrichment using Sodium pyruvate followed by cultivation on both EMB agar and Tryptic soy agar (TSA). All the tested VBNC cells regained its ability to grow and form colonies and this agreed with Tolba *et al*. (2020). But Gu *et al*. (2020) showed that *E. coli* O157:H7 injured via treatment with 50 mg/L free chlorine, was not able to recover after incubation (TSB supplemented with 0.3% of sodium pyruvate. sodium pyruvate, a key intermediate metabolite in glycolysis, plays a crucial role in the resuscitation of VBNC (viable but non-culturable) cells. While VBNC cells cannot grow on standard media, they can be revived on media supplemented with sodium pyruvate, i.e., simply able to grow and this agreed with Mizunoe *et al*. (1999) suggested that adding sodium pyruvate and catalase to the growth medium could protect cells that can't be cultured from oxidative stress. This would allow the cells to recover and form colonies. Sodium pyruvate act as a sort of carbon source that can utilized by the bacteria and as H_2O_2 degrading compound which could facilitate the resuscitation of VBNC cells under prolonged stress or the effect of toxic chemicals such as H_2O_2 and Imazaki and Nakaho (2009). *E. coli O157:H7* have two-component systems, BtsSR and YpdAB which respond to extracellular pyruvate, composed of a membrane-integrated histidine kinase (BtsS/YpdA) that can perceive pyruvate, and a cytoplasmic response regulator (BtsR/YpdB) mediates *btsT* expression. So, with the added of pyruvate, VBNC cells initiate DNA and protein biosynthesis for growth restoration (Behr *et al*., 2017). Another model for resuscitating *E. coli* is the removal of different VBNC-inducing factors, such as the removal of copper ions and temperature change effect. Aurass *et al*. (2011) reported that when VBNC *E. coli* was induced by copper ion as stress factor, VBNC *E. coli* was regrowth in a rich agar medium for 6 to 11 days with repeated washing with cold EDTA to remove the copper ion stress, the cells became culturable and partly formed colonies in the medium (Aurass *et al*., 2011). Resuscitation of functional bacteria such as flavor-producing strains, fermentation strains, and probiotics which entering a VBNC state makes them become a hidden resource for potential industrial application which may have great value for the food industry (Su *et al*., 2018). But the new researchers did huge effort on studying the risks of VBNC bacteria and its resuscitated cells to human health. Makino *et al*. (2000) mentioned that VBNC *E. coli O157:H7* in salted salmon roe could regain pathogenicity in germfree mice by resuscitating in the mouse intestine. Also, Liu *et al*. (2010) proved that VBNC *E. coli O157:H7* and VBNC cells retained the ability to produce toxin genes or virulence proteins.

The obtained results in Table 3 and Figure 2 indicated that there was a significant difference ($P<0.05$) between different concentrations H_2O_2 treatments: H_2O_2 addition did not able to induce the *E. coli* O157 in VBNC state at used concentration by 0.1, 0.3 and 3mM after 30 minutes of treatment. However, H_2O_2 reduced the count of *E. coli* O157 in groups G1V.GV and GV1 to 6.49±0.03cfu/ml, 6.04±0.06cfu/m and 4.97±0.01cfu/ml l, respectively in compared with C group count by 6.93±0.04cfu/ml. This finding fits with what Morishige *et al*. (2013) found: low concentrations (0.1–1 mM) of H_2O had little effect on the ability of *Salmonella* species to grow in culture, with 1 mM lowering it by only 1/10 of the control and 3 mM lowering it to about 1/10,000 of the control. Similarly, Munna *et al*. (2013) observed that while 3 mM H_2O_2 did not induce the VBNC state in *E. coli* O157 after 30 minutes but could induce the VBNC state after 36 hours of incubation. The reason for this delayed response could be that *E. coli O157* is making eicosatetraenoic acid (EPA), which might help protect against oxidative damage from H_2O.

As, we discussed above the VBNC bacteria are no longer detectable or culturable by conventual methods. So, alternate methods must be done to demonstrate whether these cells are dead or alive such as autoradiography fluorescent microscopy, flow cytometry and real-time,

reverse transcriptase (RT) polymerase chain reaction (PCR) (Ramamurthy *et al*., 2014). However, it is not possible to distinguish dead bacteria from live bacteria by normal PCR or qPCR because these methods only target DNA. So, propidium monoazide (PMA) was used in real-time PCR to measure the DNA of living cells, as PMA enter via the damaged cell membrane of dead bacteria and react with the hydrocarbon portion of this cell DNA, applying structural changes in DNA. So, dead cell DNA cannot replicate in the PCR reaction, and only alive cell DNA can replicate (Wideman *et al*., 2021). PMA and EMA have been improved and used successfully to find different types of bacteria, such as enteropathogenic *E. coli O157*, Salmonella, and *L. monocytogenes*, in food, environmental samples, and pure cultures (Nocker *et al*., 2007). In this study, *E. coli* O157 cells were induced into the VBNC state using different concentrations of chlorine. As a result, the treated samples contained a mixture of viable and non-viable cells. To detect this mixture, PMA-qPCR was employed. This technique selectively penetrates dead cell membranes and prevents the amplification of DNA from dead bacteria, allowing only the amplification of sequences from viable cells. The *rfbE* gene, which is a good indicator of living *E. coli O157* cells, was used to make sure that there were living cells (Lin *et al*., 2017).

Specifically, the fold change decreased 3.96-fold in Group II (treated with 200 ppm chlorine), 2.52-fold in Group III (treated with 100 ppm chlorine), and 1.77-fold in Group IV (treated with 50 ppm chlorine) compared to the control group. The PMA-qPCR method could only detect about 3 log CFU/mL of VBNC cells. This is like what Xiao *et al*. (2013) found, which was that PMA-qPCR could only measure up to 10^2 CFU/mL. Therefore, developing accurate and efficient methods for detecting VBNC *E. coli* O157 in food products is essential and remains necessary for microbial research. Also, *E. coli* cells could induce to VBNC state due to high carbon dioxide pressure and was stated that the sod gene increased significantly in transcriptomic studies, but its expression changed insignificantly via analyzed protein (Zhao *et al*., 2016). From the food safety aspect concerned with VBNC state, in 1998, an EHEC O157:H7 outbreak in salted salmon roe occurred in Japan. It was demonstrated that patient samples containing O157:H7 could not grow on conventual culture media after incubation in 13% NaCl, but after being resuscitated in yeast extract broth, 90% of the VBNC cells were shown to be viable state (Arana *et al*., 1992).

CONCLUSIONS AND RECOMMENDATIONS

Sanitizers employed for the cleaning and disinfection of food contact surfaces must be utilized at adequate concentrations. Chlorine facilitates the induction of *E. coli O157:H7* into a viable but non-culturable state, whereas hydrogen peroxide only diminishes the number of viable cells. When utilized at low concentrations, it induces a viable but non-culturable (VBNC) condition that does not proliferate on culture media. The study determined that the induction, identification, and control of VBNC *E. coli O157:H7* in the food system is crucial to mitigate possible threats to human health. VBNC pathogenic bacteria present a potential threat to the food industry as they do not proliferate on standard microbiological media, allowing them to elude identification in conventional plating studies. *E. coli O157:H7* has been documented to reach the viable but non-culturable (VBNC) stage under various environmental stressors and to resuscitate under favorable conditions, potentially leading to human infections. The PMA-qPCR technique described in this study offers a quick, sensitive, and specific method for quantifying levels of VBNC *E. coli O157:H7* on food contact surfaces, hence potentially reducing the dangers associated with the consumption of food contaminated by bacteria in this state.

NOVELTY STATEMENT

VBNC pathogenic bacteria pose a potential risk to the food industry because they do not multiply on routine microbiological media and thus can evade detection in conventional plating methods. *E. coli* O157:H7 has been found enter VBNC state under many environmental stress factors and to resuscitate under favorable conditions. Where, become a potential cause of human infections. PAM-q PCR developed in the study provide a rapid, sensitive, and specific methods to determine levels of VBNC *E. coli* O157:H7 in food contact surfaces, which potentially decreases the risks a combined to consumption of products contaminated by pathogen in VBNC state.

AUTHOR'S CONTRIBUTION

NAE, ATT and HRG conducted the study, laboratory investigation and analyzed the data and interpretation of results . All the authors wrote ,revised the original draft approved the final version of the manuscript.

Ethical guideline

The study was conducted according to the ethical guidelines of *Animal Health Research Institute (AHRI)* in regarding the handling and disposal of VBNC pathogens.

Conflict of interest

The authors have declared no conflict of interest.

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