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Review

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Significance of Viable but Nonculturable *Escherichia coli*: Induction, Detection, and Control

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Diseases caused by foodborne or waterborne pathogens are emerging. Many pathogens can enter into the viable but nonculturable (VBNC) state, which is a survival strategy when exposed to harsh environmental stresses. Pathogens in the VBNC state have the ability to evade conventional microbiological detection methods, posing a significant and potential health risk. Therefore, controlling VBNC bacteria in food processing and the environment is of great importance. As the typical one of the gram-negatives, *Escherichia coli* (*E. coli*) is a widespread foodborne and waterborne pathogenic bacterium and is able to enter into a VBNC state in extreme conditions (similar to the other gram-negative bacteria), including inducing factors and resuscitation stimulus. VBNC *E. coli* has the ability to recover both culturability and pathogenicity, which may bring potential health risk. This review describes the concrete factors (nonthermal treatment, chemical agents, and environmental factors) that induce *E. coli* into the VBNC state, the condition or stimulus required for resuscitation of VBNC *E. coli*, and the methods for detecting VBNC *E. coli*. Furthermore, the mechanism of genes and proteins involved in the VBNC *E. coli* is also discussed in this review.

Keywords: *Escherichia coli*, viable but nonculturable state, resuscitation, mechanism, detection methods

Introduction

Public health concerns regarding microbial food safety have been increasing worldwide in recent years, accompanied by risks of deadly foodborne diseases. For example, the incidence of illnesses or death caused by major known pathogens has increased worldwide [1, 2]. It is estimated that nearly 9.4 million illnesses, 55,961 hospitalizations, and 1,351 deaths are caused each year by consuming food contaminated with 31 known pathogens of foodborne disease in the United State, including *Campylobacter* spp., nontyphoidal *Salmonella* serovars, and *Escherichia coli* [2–4]. Furthermore, there are several sources of transmission of microbial outbreaks, including food, animal contact, person-to-person contact, and water [5]. Infections or outbreaks caused by pathogenic *E. coli* can be a result of consumption of contaminated foods, including beef, milk products, fresh vegetables, and contaminated water [1, 6]. Therefore, it is important to monitor and control the major known pathogens in food processing and environmental conditions. Stresses are present in the natural environment as well as during food processing, which is a great challenge for microbes to survive and construct networks (repair systems) for encountering these adverse conditions [7]. Accordingly, microbes have evolved unique adaptive strategies to combat these adverse and changing environments and maintain survival under hostile conditions. It is known that bacteria could enter into the viable but nonculturable state (VBNC) when exposed to extreme conditions [8], in which the bacteria cannot grow on conventional media and thus

easily evade the conventional detection methods, causing false-negative results. The public health significance of VBNC cells have been discussed by Fakruddin *et al.* [9]. Consequently, the VBNC bacteria pose a potential health risk.

In 1982, Xu *et al.* [10] first defined the VBNC state of *E. coli* and *Vibrio cholerae*. The VBNC state of bacteria exhibits a pattern similar to dormancy [8], in which bacteria cannot form a colony in standard medium but can retain their metabolic activity and express toxic proteins [8, 9, 11, 12]. To date, more than 60 bacterial species have been verified to enter into the VBNC state [11, 12], including *Escherichia, Vibrio,* and *Listeria*. Adverse conditions, such as low temperature [13, 14], radiation [15, 16], sea water [17, 18], and starvation [12, 19], can cause bacteria to enter into the VBNC state. With regard to viability and nonculturability, VBNC cells have the ability to evade adverse environmental stresses. In some ways, the VBNC state is a strategy of adaptation to an adverse environment. In addition, resuscitation is also an advantageous characteristic of VBNC cells. The VBNC bacteria can be cultured again when placed in an environment with appropriate nutrition [20] and proper temperature [8, 11]. VBNC cells have some specific characteristics. Compared with dead cells, VBNC cells have an intact membrane, successfully maintain gene expression, and avoid cytoplasmic leakage. In contrast, dead cells have a damaged membrane and are metabolically inactive. Similarly, compared with viable and sublethally injured cells, VBNC cells cannot grow in standard medium, which is a specific character of VBNC cells. Additionally, there are physiological and molecular differences between VBNC cells and viable, culturable cells, including cell wall and membrane compositions, gene expression, adhesion properties, virulence potential, metabolism, cellular morphology, and physical and chemical resistance [21, 22]. For example, cell size reduction has been found in VBNC *E. coli* [23], and cell dwarfing and round cells have been described for some VBNC cells [24, 25]. Moreover, VBNC cells have low metabolism[8] and a high level of ATP [26]. However, the mechanism by which bacteria adjust in the VBNC state remains unclear. Thus far, some regulators and effectors have been found to be related to the induction and resuscitation of VBNC bacteria [21], including alkyl hydroperoxide reductase subunit C (ahpC1 and ahpC2), the catalase KatG, glutathione S-transferase (GST), a LysR-type transcriptional regulator (OxyR), resuscitation-promoting factors (Rpfs), and the stress regulator RpoS [11, 27–29]. Besides the stress regulator RpoS, outer membrane proteins (EnvZ/OmpR) have also been found to be involved in the

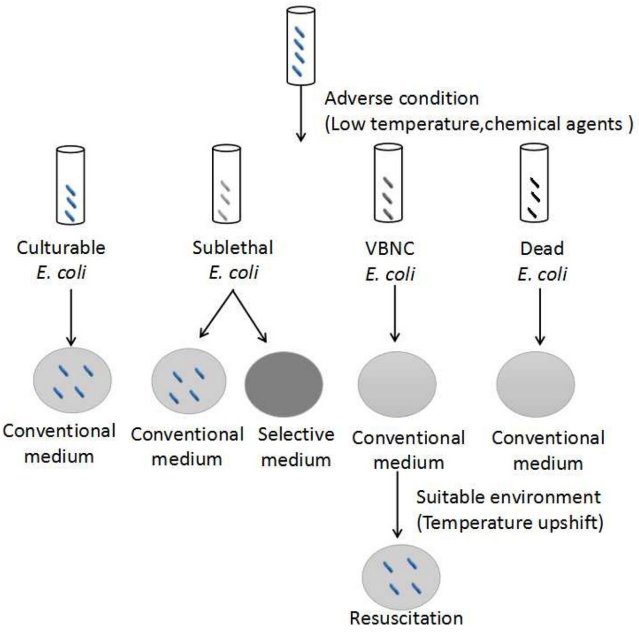

Fig. 1. The four states of *E. coli* under extreme conditions.

VBNC state [21, 30].

It is very important to control the infection of foodborne pathogens during food processing. Exposure to adverse conditions such as heating, freezing, drying, freeze-drying, irradiation, high hydrostatic pressure, fermentation, or the addition of antimicrobials and chemicals may result in different states of bacteria, including dead cells, sublethally injured cells, and normal cells [31]. With the finding of the VBNC bacteria, there is the increasing chance of inducing them when exposed to adverse conditions, and thus more attention should be paid to the VBNC bacteria. Fig. 1 depicts the four possible states of *E. coli* when exposed to stressful conditions or disinfection treatment; namely, culturable *E. coli*, sublethally injured *E. coli*, VBNC *E. coli,* and dead *E. coli*. In recent years, *E. coli* strains have also been found to have the ability to enter the VBNC state under certain adverse conditions [8], including food processing, heating, low temperature, or radiation [16, 32]. VBNC *E. coli* cannot grow on routine medium, and can recover from the nonculturable state when the growth condition is favorable, which is called resuscitation. In addition, owing to the viability of VBNC *E. coli*, it is difficult to distinguish them using conventional methods based on the plate counting method, yet resuscitation of VBNC *E. coli* can result in possible infection of humans or other living organisms [33]. Therefore, the presence of VBNC *E. coli* makes it more difficult for monitoring pathogenic *E. coli*, and is likely to produce false-negative results. Despite the

fact that there are a few reports about diseases caused by VBNC *E. coli*, more attention should be paid to the health risks of VBNC *E. coli*, especially pathogenic VBNC *E. coli*. In fact, investigation of the induction, resuscitation, mechanism, and detection of VBNC *E. coli* has an instructive significance for further research of other VBNC bacteria.

The purpose of this review was to highlight the possible risk and impact on public health of VBNC *E. coli* and guide the production and pathogen testing for the food industry. In addition, some valuable information may be provided for environmental and medical areas. As the model of the gram-negatives, many characteristics of VBNC *E. coli* are explained in this review, including the inducing factors, resuscitation strategy, proteins and genes involved in the VBNC *E. coli* mechanism, and detection methods for VBNC *E. coli*, which may give a reference of other gram-negative bacteria. In addition, the health risks of VBNC *E. coli* in the environment and food processing areas are discussed.

Health Risk of VBNC *E. coli*

E. coli, a typical gram-negative bacterium, consists of a diverse group of strains, including enteropathogenic *E. coli*, enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli*, enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli*, and diffusely adherent *E. coli* [34]. *E. coli* cannot produce spores and can be used as the indicator for testing the quality of water. Pathogenic *E. coli* is one of the most widespread foodborne and waterborne bacteria, which can contaminate water and soil through human and animal feces or organic fertilizer and sewage [35]. In 2011, a number of hemolytic uremic syndrome (HUS) and bloody diarrhea cases were reported in Germany, which were caused by a large outbreak of *E. coli* O104:H4, a strain expressing genes characteristic of both EHEC and EAEC [36–40]. Several evidences point to the fenugreek sprouts and seeds, which are considered as the contamination source related to the 2011 outbreak in Germany [41]. Moreover, as few as 10 cells of pathogenic *E. coli* can cause infection, which poses a threat to the environment and human health [35]. Therefore, it is necessary to monitor the distribution of pathogenic *E. coli* at important stages of the food chain, from agricultural production on the farm to processing, manufacturing, and preparation of foods.

Furthermore, *E. coli* strains have been demonstrated to have the ability to enter into the VBNC state [8]. Aurass *et al*. [42] studied the EHEC/EAEC O104:H4 strain, which was linked with the 2011 German outbreak of HUS, and confirmed its ability to enter into the VBNC state. Pathogenic VBNC *E. coli* strains have been implicated in a variety of diseases. When in the VBNC state, pathogenic bacteria can be nontoxic. However , the virulence can be regained after resuscitation into culturable cells under suitable conditions, which brings potential health risk human beings. In addition, Liu *et al*. [43] found higher expression of *stx1* and *stx2* by RT-PCR when *E. coli* O157:H7 was in the VBNC state than the culturable state. As biotic and abiotic ecological factors and hostile environments exist in rivers and oceans, allochthonous *E. coli* may gradually develop the VBNC state to resist the unsuitable conditions [44, 45]. In food processing, the interaction between extrinsic or environmental factors and intrinsic factors can be the inducing factors of VBNC *E. coli*. As the VBNC *E. coli* cells cannot be detected using conventional plate counting method, it is more likely to evade the detection of VBNC pathogenic *E. coli* and cause a false-negative result, which poses a potential threat for food safety and human health. Thus, VBNC *E. coli* should be taken into account when detecting pathogens.

Inducing Factors of VBNC *E. coli*

Food is a complex system, in which the nutrients available can promote the growth of bacteria. In addition, the natural environment is also a large vessel that can breed a variety of microorganisms. Many stressful conditions can cause *E. coli* to shift to the VBNC state, including changes in temperature [46], pH [30], sea water [17], copper ions [42], visible light irradiation [45], lack of nutrition [11, 47], and plasma treatment [48, 49], which are shown in Table 1.

Table 1. Typically reported VBNC *E. coli*-inducing factors.

Inducing factors	Conditions	References
High-pressure	5 Mpa	[52]
Carbon dioxide	Nisin 200 ppm, 10 Mpa	[23]
Radiation	UV (60–90 mJ/cm^2)	[16]
	Plasma treatment	[49]
	TiO_2 photocatalysis	[50]
Water	River water	[57]
	Sea water	[17]
	Deionized water	[43, 87]
	Chlorination of water	[43, 65, 88]
	Anaerobic digested sludge	[79]
Copper ions	500 mM $CuSO_4$	[42, 54]
Oxidative stress	H_2O_2	[30, 59, 68, 89]
Low temperature	4°C	[90]
	8°C	[43]
Starvation	Lack of nutrition	[61, 65, 87]

Nonthermal Treatment

Nonthermal treatment, as a mean of sterilization, inactivates and induces VBNC *E. coli* during food processing and other disinfection processes. One of the most widely used disinfection and sterilization technologies, radiation, including UV light [16], TiO_2 photocatalysis [50], and plasma treatment [49], has also been demonstrated to induce the formation of VBNC *E. coli*. Said *et al*. [51] showed that a UV doses equal to 60 and 90 mJ $cm^{2\,-1}$ was able to induce the VBNC state in the majority of *E. coli* cells and lose their cultivability with and without subsequent reactivation, respectively. Meanwhile, based on the *E. coli* UV-inactivation kinetic curve, 99.99% of *E. coli* can lose the colony-forming ability after exposure to 45 mJ $cm^{2\,-1}$ of UV light. Kacem *et al*. [50] demonstrated that TiO_2 (1.66 g/l)-mediated photocatalysis could induce the VBNC state in *E. coli*. Zhao *et al*. [52] found that *E. coli* O157:H7 could be induced into the VBNC state after high-pressure CO_2 (HPCD) treatment at 5 Mpa and 25°C, 31°C, 34°C, and 37°C, with nearly an 8 log reduction in 40, 30, 28, and 25 min, respectively. The results demonstrated that radiation and HPCD treatment can induce *E. coli* into the VBNC state. HPCD treatment and radiation have been employed for pasteurization in the food industry, such as environmental disinfection and pasteurization of liquid foods, since the technique can retain the flavor and structure of food and is operational. However, the finding of VBNC *E. coli* has future public health implication, indicating that products disinfected by HPCD treatment should be checked for VBNC populations of bacteria using molecular-based methods to ensure the safety of the product.

Nonthermal treatment, as a mean of disinfection, has been widely used in the food industry since it can retain better flavor and nutrition of the food compared with thermal treatment. However, nonthermal treatment could induce the formation of VBNC *E. coli* cells rather than kill them, which poses a future public health risk. Different treatment times and conditions may result in different states of bacteria. Exploration of the relationship between the nonthermal treatment conditions and the ratio of death of *E. coli* and VBNC *E. coli* is the key point for processing foods to guarantee food safety. Therefore, the processing conditions suitable for induction of VBNC *E. coli* should be avoided as far as possible.

Chemical Agents

E. coli strains could be isolated from cooked and raw meat samples and also from environmental samples, which involve human infections. Although *E. coli* can form biofilm to cope with the adverse conditions, which acts as a reservoir for pathogens, there are indications that the biofilm populations of pathogenic bacteria, including *E. coli*, persist in the VBNC state and remain unnoticed [53]. Meanwhile *E. coli* cannot avoid being in contact with chemical substances in the environment. Some chemical agents also induce VBNC *E. coli*, including copper ions [54], hydrogen peroxide (H_2O_2), and disinfectants (chlorine). Copper ions can accelerate the intracellular redox and increase the toxicity of copper ions [55]. Aurass *et al*. [42] found that copper ions could facilitate *E. coli* entry into the VBNC state; under copper ion stress at 4°C, the majority of cells entered into the VBNC state; in contrast, cells without copper ion stress at 23°C remained culturable for at least 40 days [42]. H_2O_2 is a frequently used substance in the food industry. Munna *et al*. [56] studied the culturability of *E. coli* cells in the late log phase in the presence of 3 mM H_2O_2 and observed that the cells started to enter into the VBNC state after 36 h of incubation. Meanwhile, *E. coli* O157:H7 cells were induced into a VBNC state after the treatments of sterilized deionized water and sterile chloraminated tap water at 4°C [32], which contributed to the effect of chlorine. However, the use of disinfectants and preservatives is inevitable in the food industry. Therefore, the finding of VBNC *E. coli* in tap water warrants more attention due to its potential public health risk.

Environmental Factors

As *E. coli* strains exist in complicated environments, they are sensitive to the changing conditions. It has been found that river water [57], starvation, oxidative stress, and low temperature can induce *E. coli* cells to enter into the VBNC state. Dinu and Bach [58] studied VBNC *E. coli* O157:H7 in the phyllosphere of lettuce by inoculating the strain onto the lettuce at 4°C. The authors found that when the temperature was increased to 8°C, the *E. coli* cells maintained stable GFP expression during the VBNC response, and these bacterial populations evolved toward the VBNC state. Asakura *et al.* [59] found that oxidative stress induced different *E. coli* strains toward the VBNC state. Lothigius *et al*. [17] successfully induced the formation of VBNC *E. coli* cells in sea water and freshwater. Muela *et al.* [45] reported that combining starvation, sea water, and visible radiation enhanced the rate of VBNC *E. coli*, indicating that a combination of multiple factors may have a stronger effect than a single factor.

Water systems, including rivers, sea water, and tap water, are a multiple system. Important waterborne bacterial pathogens can infect the gastrointestinal tract of humans

and warm-blooded animals and contaminate the water system by excreting them with the feces into the environment. The presence of VBNC cells in water (and food) has significant consequences, including that *E. coli* cannot be used as an indicator of fecal contamination when the cells are in the VBNC state. The existence of VBNC *E. coli* aggravates the contamination of the water system, which brings potential health risks.

Resuscitation of VBNC *E. coli*

Under certain conditions, the VBNC bacterial cells can regain normal metabolic activity and become culturable again in conventional medium, which is defined as resuscitation. In addition, suitable conditions for the VBNC bacteria could be supplied during the food processing that promote the resuscitation of VBNC cells. As the resuscitated VBNC cells have metabolic activity and can express the toxic proteins, it brings a big challenge and huge health risk for human beings. Although examination of the survival aspect of the VBNC state has frequently been reported for various bacteria, resuscitation has been most extensively studied in *E. coli*. Researchers have successfully achieved resuscitation of VBNC *E. coli* by shifting the *E. coli* cells to a more appropriate temperature or nutrient-rich medium and by addition of beneficial chemicals [8, 11, 17, 46, 60–62]. Resuscitation stimuli for VBNC *E. coli* cells are summarized in Table 2.

The removal of various VBNC-inducing factors is commonly used for resuscitating *E. coli*, such as the removal of copper ions and temperature upshift. Aurass *et al*. [42] observed that when copper ion-stressed VBNC *E. coli* was placed in a rich agar medium for 6 to 11 days of incubation accompanied by repeated washing with cold EDTA to relieve the copper ion stress, the cells became culturable and partly formed colonies in the medium. Zhao *et al*. [52] reported that HPCD-induced VBNC *E. coli* O157:H7 could be successfully resuscitated by upshifting the incubation temperature to 37°C , which indicated that improving the incubation temperature could help resuscitate VBNC *E. coli*.

Table 2. Reports of resuscitation stimuli of VBNC *E. coli* cells.

Resuscitation stimuli	References
Sodium pyruvate	[27, 59]
Amino acids	[61]
Heat-stable enterobacterial autoinducer	[63]
Supernatants of growing cells	[61]
Temperature upshift	[16, 61]
Relief from saline stress	[91]

In recent studies, the addition of substances to inactivate cells has been used to improve the resuscitation of VBNC *E. coli*. In particular, several studies have reported the beneficial effect of adding sodium pyruvate, which can eliminate oxidative stress. In one study, oxidative stress-induced *E. coli* cells were placed in TSA medium containing 0.1% sodium pyruvate (TSA-SP) and in TSA medium, and the cells in the TSA-SP medium were found to have a shorter survival time than the cells in the TSA medium [59]. The oxidative stress-induced *E. coli* cells in the TSA-SP medium also showed a better resistance to oxidative stress. A similar conclusion was reached in another study [27]. These findings indicate that sodium pyruvate is a good supplement for protection against oxidative stress and resuscitation from the VBNC state. In addition, Pinto *et al*. [61] demonstrated that some amino acids were involved in the resuscitation of VBNC *E. coli*, and they confirmed that VBNC *E. coli* cells induced by stresses could be resuscitated in a limited time. A heat-stable enterobacterial autoinducer (AI) also has an important function in surviving VBNC *E. coli*. Reissbrodt *et al*. [63] found that nutrient-rich p-TSB medium supplemented with enterobacterial AI promoted the resuscitation of stressed *E. coli* O157:H7 cells for significantly longer periods of time.

So far, how the VBNC *E. coli* cells adjust themselves to resuscitate from the VBNC state and recover culturability still remain unknown. A hypothesis of a resuscitation model has been put forward by Epstein [64], who first proposed the concept of scout cells, which can sense a changing incubation environment to help determine resuscitation or maintenance of the VBNC state. This is an interesting hypothesis that may help guide inquiry of resuscitation factors for VBNC *E. coli* because not all cells are successfully resuscitated [48, 65], and some of the VBNC cells may finally die and others may persist in the VBNC state for a long time. It is noted that the resuscitation of the VBNC cells makes the cells regain virulence and secrete toxic protein, which increase the health risk. The resuscitation stimulus of VBNC *E. coli* can be used as guidelines to study the conditions of other gram-negative bacteria.

Functional Genes and Proteins of *E. coli* in the VBNC State

E. coli, in particular serovar O157:H7, is an important foodborne pathogen that represents an increasingly

significant public health issue. Moreover, VBNC *E. coli* can retain metabolism [11], express genes [66], and regain culturability under proper conditions [61]. Thus, the VBNC state of *E. coli* increases the potential risk of outbreaks of foodborne illness, which prompted researchers to study the mechanism of VBNC *E. coli*. At present, the mechanism of how VBNC *E. coli* adjusts itself still remains unclear. However, some researchers have studied the genes and proteins involved in the VBNC *E. coli*. It has been reported that the Rpf protein of *Micrococcus luteus* can promote the resuscitation of VBNC cells [67]. Nonetheless, reports on the mechanism by which VBNC *E. coli* regulates itself are scarce. To date, Rpos, EnvZ [30], and OmpW [59] have been shown to be involved in the VBNC state of *E. coli*. Genes and proteins involved in the regulation of VBNC *E. coli* are listed in Table 3.

Studies have been conducted on genes and proteins involved in the underlying mechanism of the VBNC *E. coli*. Desnues *et al*. [68] found that specific proteins were carbonylated in non-culturable *E. coli* cells. The levels of stress regulons, such as σ^S, in non-culturable *E. coli* populations were upregulated, and enzymes responsible for the elimination of oxidative stress like, RpoS and Sod, were found to be inactivated in nonculturable cells owing to the oxidative damage. RpoS is responsible for the increase in membrane resilience and regulates the synthesis of trehalose that protects proteins from denaturation by heat and may also protect against pressure in *E. coli* [69]. Comparison of *rpoS* mutant (gene inactivated) strains with the parental strains demonstrated that RpoS protein was related to VBNC *E. coli* [27]. It was also found that *rpoS* mutant *E. coli* cells lost their culturability in only 21 days when incubated in an artificial oligotrophic medium at 4°C, whereas the parental strain required 33 days to lose culturability, which indicated that the *rpoS* gene is involved in regulating VBNC *E. coli*. The small nucleotide guanosine tetraphosphate ppGpp has also been shown to be regulated in a manner similar to RpoS. Nucleotide ppGpp plays an important role in redirection of transcription and thus the important genes for starvation survival and virulence are favored at the expense of those required for growth and proliferation [70]. Moreover, *rpoS* mutant *E. coli* cells have a low resuscitation ability, which results in a short period in the VBNC state, a phenomenon that was observed in *Salmonella enterica* as well [71].

It appears that EnvZ, a sensor protein with both kinase and phosphatase activities, also regulates VBNC *E. coli* [30]. The researchers placed an EnvZ-deficient strain in sea water and exposed the cells to osmolarity and pH stress to induce the cells to enter the VBNC state. They concluded that the EnvZ-deficient strain could not enter the VBNC state owing to the disability of sensing environmental changes. Furthermore, it was found that this mutant strain had a reduced capacity to survive under the stressful conditions, which indicated that the loss of the EnvZ protein had no effect on survival. Probably the most important reason for this phenomenon is that the EnvZ-deficient strain lost the ability to sense the changes in environment and this interfered with the entry into the VBNC state. In addition, EnvZ itself was not influenced by pH and osmolarity, which indicated that EnvZ itself was involved in the entry of VBNC *E. coli*.

Meanwhile, maintaining the integrity of the plasma membrane is the key factor for bacteria to exist in the VBNC state. The outer membrane, as the first barrier for gram-negatives, plays a significant role in sensing the environment and taking up small charged or uncharged molecules into the bacterial cells. As UV disinfection does not destroy the membrane and the membrane remains intact, *E. coli* can be resuscitated from the VBNC state when supplied the proper nutrients and temperature [16]. Asakura *et al*. [59] found that the expression level of outer membrane protein W (OmpW) was related to VBNC *E. coli* O157:H7; the decreased expression of OmpW may induce

Table 3. Functional genes and proteins regulated in VBNC *E. coli*.

Genes and proteins	Function	References
Rpos	Hinders the induction of VBNC *E. coli*;	[27, 69]
rpos	Responsible for the increase in membrane resilience and regulates the synthesis of trehalose	
ppGpp	Plays a role in redirection of transcription;	[70]
	Hinders the induction of VBNC *E. coli*	
EnvZ	A sensor protein;	[30]
	Senses changes in the environment and interferes with the induction of VBNC *E. coli*	
Outer membrane protein W (OmpW)	Regulates the induction and resuscitation of VBNC *E. coli*	[45, 59]

E. coli O157:H7 into the VBNC state, whereas the overexpression of OmpW may hinder the recovery of *E. coli* under stress, indicating that the changes in the composition of the cell membrane can lead to better adaptation to the environment [45].

At present, the mechanisms by which *E. coli* regulates itself in the VBNC state are still poorly understood. Thus, more research studies employing advanced technology, such as whole-genome sequencing and proteomics, are still needed to elucidate the mechanisms of the VBNC state.

Methods for the Detection of VBNC *E. coli*

The hallmark characteristic of VBNC cells is nonculturability [12, 14]. However, metabolic activity in VBNC *E. coli* has been reported [59], which shows that nonculturable does not mean dead. In particular, *E. coli* O157:H7 can also express virulent genes *stx1* and *stx2* in the VBNC state [17], which is a potential health risk for humans. As traditional detection methods cannot distinguish VBNC *E. coli* from dead cells and VBNC *E. coli* cannot grow in conventional culture medium, a main focus of current research is how to effectively and rapidly detect VBNC *E. coli*. Commonly used detecting methods are listed in Table 4.

Staining Detection-Based Methods

Staining, a basic step for determining viability, is the most useful method for detecting VBNC *E. coli*. The principle of staining is based on the integrity of the cell membrane, as dead cells have a damaged membrane and VBNC and viable cells an intact membrane [8]. The LIVE/DEAD Baclight staining assay (SYTO9/propidium iodide) offers advantages in this regard [72]. There is a competitive relationship between the two dyes. SYTO9, a viable cell indicator, can penetrate the cell membrane, staining the cells green; propidium iodide can only penetrate the damaged cell membrane, staining dead cells red. Therefore, with the help of flow cytometry or fluorescent microscopy, viable cells can be distinguished from dead cells based on color. The LIVE/DEAD Baclight staining assay has been widely used in the detection of VBNC [42, 52, 73]. In addition, acridine orange and DAPI, as other dyes, can also be used to distinguish dead cells and viable cells, based on the morphology of the bacteria after staining to distinguish the viable cells from dead cells and the counting done under the fluorescent microscope [74].

Flow cytometry (FCM), a rapid technique, has been used to enumerate and determine optical and fluorescent properties of particles [75]. In recent years, FCM has been applied in microbiology in which its central role is its ability to generate population statistics and determine bacterial viability and physiology, which does not rely upon microbial growth for analysis, allowing detection of bacteria that are unable to grow on agar plates, the VBNC phenotype. Flow cytometric analysis has been described as a method that allows for the selective detection of only viable cells. Anvarian *et al*. [76] used the total viable count (TVC) and flow cytometric analysis to investigate the effects of orange juice clarification on the physiology of *E. coli* K-12. Dyes Bis-oxonol (BOX) and PI were used in the flow cytometric analysis. BOX only enters cells with a collapsed membrane potential, signifying injury. PI, a red

Table 4. Common used for detecting methods VBNC *E. coli*.

Method	Concrete	Principle	Significance	References
Staining	LIVE/DEAD Baclight assay (SYTO9/PI) DAPI	SYTO9 strains the viable *E. coli* in green; propidium iodide (PI) stains the dead *E. coli* in red	Presents the result in a straightforward way	[42, 52, 73]
	Acridine orange	Use together with flow cytometry or electron micoarray		
DVC (direct viable count)	DVC-FISH	Direct viable count combined with fluorescence in situ hybridization	Presents the result in a straightforward way	[30, 57, 92]
PCR	PMA-PCR	PMA combined with DNA in cells that have a damaged membrane, which inhibits PCR amplification or DNA loss during DNA extraction	Monitors the specific genes, like *rfbE*, *fliC*, *rpoS* in *E. coli* cells	[16, 17, 32, 46, 50, 84, 86, 87, 90, 93]
	EMA-PCR	Based on EMA, which is a DNA binding dye	Accurate and fast	
	RT-PCR RT-qPCR	DNA exists in both live and dead bacteria, whereas mRNA has a very short half-life in dead cells		
Fluorescent microscopy		Used together with staining method	Present the result in a straightforward way	[50, 87]
Flow cytometry		Used together with staining method	Fast and accurate	[76]

DNA dye, can only enter cells with compromised membranes, thus indicating dead cells. Compared with TVC, flow cytometric analysis has an accurate detection limit and can reveal the presence of VBNC *E. coli* in orange juice, and this method is both fast and convenient for the purposes of detection. Direct viable cell counting is also another method that can be applied with staining [77].

The application of staining in detecting VBNC *E. coli* has shown great advantages. Staining is a straightforward way to display the proportion of viable and dead cells and can detect the membrane integrity of VBNC cells. Furthermore, it can be used to observe the dynamic changes of dead and viable cells in a constant time.

Molecular Detection-Based Methods

VBNC bacteria cannot form colonies in routine medium and thus result in the underestimate of viable bacteria. In order to reduce potential outbreaks of infectious disease, additional molecular tests would be required when there is a chance of VBNC pathogens.

Real time-polymerase chain reaction (RT-PCR) technology is often used for detecting VBNC *E. coli*, which is based on the principle that mRNA just exist in viable cells. The greatest advantage of mRNA as an indicator is that it has a very short half-life; thus, it can be used to distinguish between viable and dead bacteria [78]. Meanwhile, RT-qPCR can achieve good quantitative detection [79], which has important significance for identifying pathogenic bacteria.

Yaron and Matthews [84] conducted an experiment to verify which gene is suitable as an indicator gene. *rfbE* [80], *fliC* [81], *stx1* [82], *stx2* [82], *mobA*, *eaeA* [83], *hly*, and 16S rRNA have been detected under various conditions using RT-PCR, and the authors concluded that *rfbE* was the best indicator for monitoring *E. coli* viability under different treatment conditions, both saving time and improving detection accuracy [84]. Liu *et al*. [43] combined RT-PCR with the fluorescent microscopy technique, and used as markers mRNAs of *rfbE* and *fliC* of *E. coli* O157:H7 to detect the VBNC state of *E. coli* in environmental water samples. The application of RT-PCR for VBNC *E. coli* detection allows for advances in monitoring gene expression under different phases.

Viability qPCR with the use of propidium monoazide (PMA) was first put forward by Nogva *et al*. [85]. PMA can enter cells with damaged membranes, but not viable cells, and then combine with DNA, and is thus a good way to monitor dead cells. PMA-qPCR can also be applied for detecting VBNC *E. coli*. Kacem *et al*. [50] combined PMA with qPCR to evaluate the inactivation of *E. coli* by TiO_2-mediated photocatalysis. Truchado *et al*. [86] detected and validated the inactivation of *E. coli* in water samples by PMA-qPCR, generating a quantitative analysis.

Considering the prominent feature (nonculturability) of the VBNC cells, alternative methods must be employed to demonstrate whether or not these cells are viable. Generally, the staining and PCR detection-based methods can determine the viability of the cells. However, the plate counting result is also the key for testing VBNC cells, which signifying the culturable cells. The VBNC cell number can be obtained by subtracting the viable cell number and the culturable cell number. Fig. 2 shows the basic step of determining the VBNC *E. coli* number based on the staining method. Improving the detection limit and shortening the detection time for VBNC *E. coli* should be the focus of future testing.

In conclusion, as the typical one of the gram-negatives, VBNC pathogenic *E. coli* strains pose a potential health risk, which should attract our attention and concern. VBNC pathogenic *E. coli* may be present in food or water and can retain metabolic activity and express toxic proteins. However, VBNC *E. coli* cannot form colonies in routine medium and thus can avoid detection via standard laboratory techniques, such as culturability. Problems will arise when the cells resuscitate from the VBNC state owing to the new culturability and continuous virulence, which threatens the health of human beings.

Considering the wide range of VBNC *E. coli*, especially pathogenic VBNC *E. coli*, in the water system and food industry, more control efforts need to be taken for eliminating

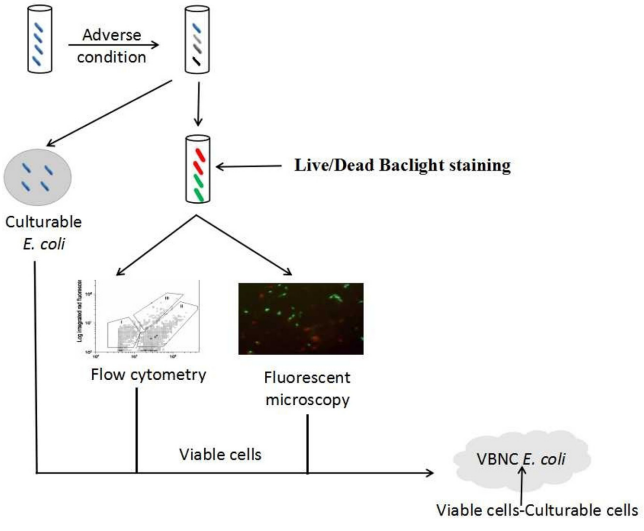

Fig. 2. Methods of detection of VBNC *E. coli* by staining.

the potential safety risks. For the moment, monitoring the VBNC *E. coli* and using effective means of sterilization are the main measures of controlling VBNC cells. Multiplex detection methods, especially molecular-based detection methods (PMA-PCR, EMA-PCR), should be employed in quality testing and bactericidal effect detection for the awareness of the existence of VBNC *E. coli* as far as possible. In addition, more rapid and sensitive methods for the detection of VBNC bacteria are imperative. In conclusion, research on the induction, detection, and control of VBNC *E. coli* is of great importance and significant to the detection of the bacteria and the avoidance of potential risks for human beings and provides reference for the other bacteria.

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