



# Induction of Viable but Non-culturable (VBNC) State in *Shigella flexneri* Under Osmotic and Nutritional Stresses at Low Temperature

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## Abstract

**Background:** Viable but non-culturable (VBNC) is a specific physiological state in which living bacteria lose the ability to grow and form colonies on conventional bacterial growth media; however, these cells remain metabolically active.

**Objectives:** Considering the importance of the pathogenicity of *Shigella flexneri* and the possibility of its transmission through contaminated water and food, we aimed to study the possibility of this bacterium entering the VBNC state under osmotic and nutritional stresses at low temperature (4°C).

**Methods:** *Shigella flexneri* was inoculated in distilled water and NaCl solutions with concentrations of 0, 5, 10, 15, 20, 25, and 30% at 4°C. The cultivability of the bacteria was checked daily. After observing the loss of cultivability, entry into the VBNC state was assessed using RT-PCR, flow cytometry, and fluorescence microscopy.

**Results:** After cultivation of *S. flexneri* in different concentrations of salt solution, the time of non-cultivability was observed at concentrations of 0, 5, 10, 15, 20, 25, and 30%, respectively, on days 19, 20, 21, 22, 22, 24, and 24. Expression results of *ipaH* and *ipaD* genes using RT-PCR, along with the presence of live cells detected by flow cytometry and fluorescent staining, indicated that the cells had entered the VBNC state.

**Conclusions:** *Shigella flexneri* can survive and enter the VBNC state under nutritional and osmotic stresses at low temperatures (4°C). In this state, although the bacterium is alive, it cannot be detected by conventional culture-dependent methods. Due to the potential risk of bacterial recovery, conventional techniques may be insufficient to detect the presence of pathogens in the VBNC state when evaluating water and food.

**Keywords:** *Shigella flexneri*, VBNC, RT-PCR, Flow Cytometry

## 1. Background

The viable but non-culturable (VBNC) state is a microbial survival strategy in response to unfavorable physicochemical conditions, characterized by the loss of culturability of microbial cells in standard culture media while maintaining their metabolic activity (1). Bacterial cells are capable of entering the VBNC state in response to various physicochemical stresses, including starvation (2), incubation outside the natural growth temperature range (3-5), elevated and lowered osmotic concentrations (6, 7), mineral salts (8), exposure to UV radiation (9), heavy metals (10), food preservatives (11, 12), etc. Unlike laboratory conditions, bacteria in nature spend most of their lives starved or nutrient-limited. Over the past two decades, microbial ecologists have

stated that less than 1% of all bacteria present in nature can grow on conventional bacterial culture media. Some believe that one reason for this is the entry of bacteria into the VBNC state (13).

*Shigella flexneri* is highly host-adapted and only infects humans and some other mammals. *Shigella flexneri* is the main agent of shigellosis (bacillary dysentery), a clinical syndrome caused by the invasion of *Shigella* species into the epithelial lining of the distal ileum, colon, and rectum (14). The World Health Organization's analysis of foodborne diseases showed that annually 600 million cases are attributed to 31 etiological agents from 2007 to 2015. Approximately 51 million people are infected with *Shigella* (15). *Shigella flexneri* is the most common species isolated worldwide (16) and accounts for 65.9% of all cases of shigellosis in a

multicenter global study (17). One of the main challenges related to this pathogen is its very low infectious dose; in humans, approximately 10-100 *Shigella* cells can cause shigellosis. However,  $9 \times 10^8$  organisms are required to produce shigellosis in rats, which indicates that humans are highly sensitive to this pathogen (18).

*Shigella flexneri* has a relatively stable genome and acquired the virulence plasmid (Virulence plasmid pINV) early in its evolution (19). The majority of the important virulence factors involved in the *Shigella* life cycle are localized to a 30 kb region termed the “entry region” on the pINV plasmid (20). This region encodes the type 3 secretion system (T3SS) and Invasion plasmid antigen (Ipa) proteins, which play a key role in initiating the invasion process and pathogenesis of *Shigella* (21, 22). Ipa proteins are identified as key virulence factors for *Shigella* infection. The T3SS injects effector proteins into host cells to manipulate the host cell machinery for the benefit of the bacterium (22). At the tip of the T3SS apparatus (T3SA) needle, the *IpaD* effector protein is polymerized as a homopentamer and is necessary for the control of virulence and secretion of effector proteins (23). Studies have shown that deletion of *ipaD* not only leads to excessive secretion of other effector proteins but also results in a non-invasive *Shigella* phenotype (24).

The *ipaD* gene is highly conserved among *Shigella* serotypes (*ipaD*: 96%) (25). The pINV plasmid contains other important genes located outside the “entry region”. The *ipaH* gene family plays a key role in the pathogenesis of *Shigella* and is responsible for post-invasion events (15). For example, the *IpaH* protein encoded by *ipaH 7.8* gene helps induce pyroptosis and escape of *Shigella* from the macrophage phagosome (26, 27). Cultivation of food is one of the main ways to detect and control microbial load, but when the bacteria enter the VBNC state, this method loses its effectiveness (28). In recent years, concerns have been raised about the possibility of regeneration and pathogenicity of VBNC bacteria. Many foods are stored for extended periods in the refrigerator (4°C), and contamination of these foods with the VBNC form of pathogens can pose risks to consumers.

## 2. Objectives

The purpose of this study was to investigate the possibility of *S. flexneri* entering the VBNC state under osmotic and nutritional stresses at 4°C.

## 3. Methods

### 3.1. Bacterial Strain

*Shigella flexneri* with PTCC number 1865 (ATCC 12022) was purchased from the Iranian Research Organization for Science and Technology (IROST).

### 3.2. Viable but Non-culturable Induction

To investigate the possibility of *S. flexneri* entering the VBNC state, different salt (NaCl) solutions with concentrations of 0, 5, 10, 15, 20, 25, and 30% were prepared. A suspension of *S. flexneri* with a turbidity of 0.5 McFarland was prepared, and 100 µL of the suspension was added to 4.9 mL of each salt solution, then incubated at 4°C. Each experiment was performed in six replicates. Cultivability was tested by daily inoculation (one loop) of each solution onto BHI (Merck; Germany) medium enriched with 0.6% yeast extract and 1% sodium pyruvate. Inoculated media were incubated at 37°C for 16 - 24 hours. Cultivation of the solutions continued until no bacterial colonies were observed on the BHI medium, which was considered the point at which the bacteria had potentially entered the VBNC state.

### 3.3. Reverse Transcriptase (RT)-PCR Method

Considering that the half-life of bacterial mRNA is 3 to 5 minutes, the expression of mRNA in the non-cultivable state indicates the survival of bacteria and their entry into the VBNC state (29). Therefore, we investigated the presence of *ipaH* and *ipaD* gene mRNA. To achieve this, 1.5 mL of each solution containing VBNC bacteria was centrifuged at 7000 rpm for 5 minutes. The supernatant was removed, and the bacterial pellets were washed three times at 7000 rpm for 5 minutes.

#### 3.3.1. RNA Extraction and cDNA Synthesis

RNA was extracted from the pellets using the DENA Zist Asia RNA extraction kit (Iran) according to the manufacturer's instructions. cDNA was synthesized using the DENA Zist Asia cDNA synthesis kit (Iran), following the manufacturer's recommendations.

#### 3.3.2. PCR Assay

A PCR assay was performed to detect *ipaD* and *ipaH*. The primer sequences used in this assay are provided in Table 1. PCR amplification was carried out in a 20 µL reaction mixture containing 5 - 7 ng of template cDNA, 10 µL Master Mix (Ampliqon; Denmark), 0.3 µM of each forward and reverse primer, and distilled water up to 20

$\mu\text{L}$ . The reaction conditions for both genes were as follows: Initial denaturation at  $95^{\circ}\text{C}$  for 4 minutes, followed by 34 cycles of denaturation at  $94^{\circ}\text{C}$  for 30 seconds, annealing at  $58^{\circ}\text{C}$  for 30 seconds, extension at  $72^{\circ}\text{C}$  for 30 seconds, and a final extension at  $72^{\circ}\text{C}$  for 5 minutes. After amplification, the products were analyzed by electrophoresis on a 2% agarose gel.

**Table 1.** Sequence of Primers Designed for RT-PCR

Primer Name	Sequence 5'→3'	Tm	Product Length (bp)
<b>ipaH</b>			
F	GTTGCTGCTGATGCCACTG	58	95
R	CCTTCTGATGCCTGATGGAC		
<b>ipaD</b>			
F	CGCTGTTCTTCCAGTCTTGC	58	206
R	CCTTGCCGATTGTTCCACCT		

### 3.4. Fluorescent Staining Assay

To evaluate the entry of *S. flexneri* into the VBNC state using a fluorescence microscope, the non-cultivable bacterial samples were centrifuged at 13,000 rpm for 10 minutes and washed three times with PBS. Untreated bacteria served as the positive control, while bacteria treated with 70% ethyl alcohol were used as the negative control. After resuspending the bacterial pellets in 100  $\mu\text{L}$  of PBS, 10  $\mu\text{L}$  of bacteria were stained with 5  $\mu\text{L}$  of fluorescent dyes, including 0.05 mg/mL acridine orange (AO, Merck) and 0.05 mg/mL propidium iodide (PI, Merck), in PBS under dark conditions for 20 minutes. Smears were prepared from the stained bacteria and observed under a fluorescence microscope (Olympus, Japan).

### 3.5. Flow Cytometry Assay

To evaluate the entry of *S. flexneri* into the VBNC state using a flow cytometry assay, the non-cultivable samples were centrifuged at 13,000 rpm for 10 minutes and washed three times with PBS. *S. flexneri* in the logarithmic phase were used to prepare negative and positive control samples, with the negative control containing live bacteria and the positive control containing dead bacteria (in terms of PI dye absorption and red fluorescence emission). Untreated bacteria served as the negative control, while bacteria treated

with 70% ethyl alcohol were used as the positive control. The bacterial pellets were then resuspended in 100  $\mu\text{L}$  of PBS, and 0.5  $\mu\text{L}$  of PI were added to the cells and incubated at room temperature for 20 minutes in the dark. Samples were analyzed using a flow cytometer (BD FACSCalibur; USA) with a single laser emitting excitation light at 488 nm (30).

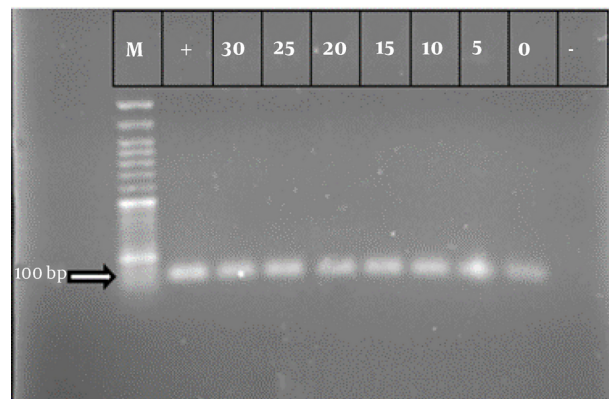
## 4. Results

### 4.1. Non-cultivable State

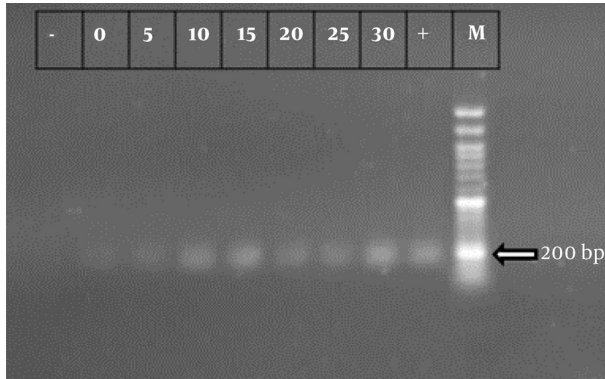
After the cultivation of *S. flexneri* in different concentrations of salt solutions, the time at which the bacteria became non-cultivable was observed at salt concentrations of 0, 5, 10, 15, 20, 25, and 30%, respectively, on days 19, 20, 21, 22, 22, 24, and 24. As the salt concentration increased, the time to non-cultivability was delayed.

### 4.2. RT-PCR

RT-PCR was performed to differentiate the non-cultivable state from bacterial death. For all treatments, the presence of mRNA related to the *ipaH* and *ipaD* genes was observed, indicating that the bacteria had entered the VBNC state (Figures 1 and 2).



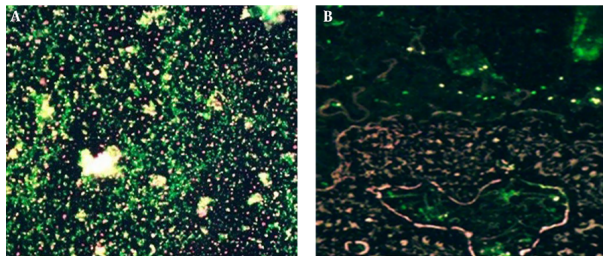
**Figure 1.** Image of *ipaH* gene electrophoresis of *Shigella flexneri*. From right to left, negative control sample, treatments 0, 5, 10, 15, 20, 25 and 30% salt, positive control sample and marker 50 pairs, respectively



**Figure 2.** Image of *ipaD* gene electrophoresis of *Shigella flexneri*. From left to right, negative control sample, treatments 0, 5, 10, 15, 20, 25 and 30% salt, positive control sample and marker 50 pairs, respectively

#### 4.3. Fluorescence Microscopic Results

Fluorescent staining of the samples on the 33rd day indicated the presence of living cells. In this case, living cells take up acridine orange (AO) and appear green, while dead cells take up propidium iodide (PI) and appear red. The presence of green cells confirms that *S. flexneri* has entered the VBNC state (Figure 3).



**Figure 3.** Florescent staining of *S. flexneri* in logarithmic phase (A) and VBNC state (B). The bacterial cells that appeared green (AO)/red (PI) when visualized under fluorescence microscopy were considered live/dead, respectively.

#### 4.4. Flow Cytometry Results

After *S. flexneri* became non-cultivable in the investigated treatments, flow cytometry was used to evaluate the bacteria's entry into the VBNC state. The analysis of the flow cytometry data revealed the presence of living *S. flexneri* cells on the 26th and 96th days of exposure to different salt concentrations (Table

2). These findings also indicated that the number of viable *S. flexneri* cells was higher in the presence of salt compared to when salt was absent.

**Table 2.** The Results of Flow Cytometry on the 26th and 96th Days After the Beginning of the Study. All, the Total Number of Cells Counted. M1, Cells Labeled with PI dye (Selective Labeling of Dead Cells)

Sample and Marker	26th Day		96th Day	
	Events	% Gated	Events	% Gated
<b>Positive control</b>				
All	10000	100.00	10000	100.00
M1	3882	38.82	4106	41.06
<b>NaCl 0%</b>				
All	3472	100.00	475	100.00
M1	970	27.94	1	0.21
<b>NaCl 5%</b>				
All	1031	100.00	198	100.00
M1	66	6.40	0	0.00
<b>NaCl 10%</b>				
All	1967	100.00	169	100.00
M1	132	6.71	0	0.00
<b>NaCl 15%</b>				
All	2870	100.00	26	100.00
M1	222	7.74	0	0.00
<b>NaCl 20%</b>				
All	3837	100.00	15	100.00
M1	303	7.90	0	0.00
<b>NaCl 25%</b>				
All	1564	100.00	8	100.00
M1	148	9.46	0	0.00
<b>NaCl 30%</b>				
All	3625	100.00	53	100.00
M1	268	7.39	1	1.89
<b>Negative control</b>				
All	6235	100.00	10000	100.00
M1	112	1.80	176	1.76

## 5. Discussion

After decades of research, the VBNC state has been recognized as an important bacterial survival strategy. A comprehensive understanding of the VBNC state and its underlying mechanisms is critical for ensuring the safety of drinking water and food. This study investigated the survival and viability of *S. flexneri* PTCC 1865 (ATCC 12022) under osmotic stress in nutrient-depleted conditions at 4°C, as food and water are typically stored at this temperature and may become contaminated from other contents in the refrigerator. The findings of this study indicated that *S. flexneri* is capable of surviving for extended periods in high NaCl concentration solutions at 4°C. Eventually, it enters the VBNC state and remains in this state for a prolonged duration.

The expression of the *ipaD* and *ipaH* genes, which are crucial for the pathogenicity of *S. flexneri*, following the bacteria's loss of cultivability, suggests their transition into the VBNC state. Moreover, the expression of *ipaD* in the VBNC state indicates the presence of the virulence plasmid *pINV* and the retention of pathogenic potential. Additionally, flow cytometry and microscopy results indicate that *S. flexneri* gradually transitions into the death phase of growth, even when exposed to high salt stress. *Shigella flexneri* can remain in the VBNC state for an extended period before entering the death phase. The results of the present study are consistent with those of other studies on this subject.

Oliveira et al. reported that *S. flexneri* 2a does not completely lose cultivability under long-term induction; however, a portion of its population enters the VBNC state. They found that short-term induction resulted in the rapid entry of *S. flexneri* cells into the VBNC state. They also suggested that low temperatures (4°C) used in nutritional and osmotic stresses can induce the VBNC state while allowing cells to maintain cultivability and viability for a longer period (31). In another study, researchers detected significant quantities of VBNC pathogenic bacteria, including *Shigella* sp., *Escherichia coli*, *Salmonella* sp., *Enterococcus faecalis*, and *Pseudomonas aeruginosa* in full-scale drinking water treatment plants. They emphasized that traditional culture-based methods are insufficient for detecting VBNC bacteria (32). Islam et al. (1993) observed that after inoculation of *S. dysenteriae* type 1 into laboratory microsomes, the bacteria became uncultivable after 2 - 3 weeks, while they entered the VBNC state and remained alive until the sixth week after the start of the study (33).

Islam et al. demonstrated that *S. flexneri* survived at pH 7.0 and 4°C in 0, 0.5, and 1% salt solutions for 27, 39, and 34 days, respectively (34). In another study, researchers revealed that after inoculating *S. dysenteriae* type 1 on some inanimate objects, the bacteria entered the VBNC state after 1.5 - 4 hours and remained detectable in this state for five days (35). Rahman et al. (1996) showed that *S. dysenteriae* type 1 can express the *stx* gene after entering the VBNC state, and the synthesized Shiga toxin retains its biological activity (36). Scientists explained that the activation of the type II toxin-antitoxin (TA) system in *Shigella* could partially maintain cell viability in the VBNC state. The TA system halts cell growth and helps *Shigella* persist under various mild stresses, such as osmotic, temperature, and nutritional stresses (37).

### 5.1. Conclusions

The results of the present study revealed that *S. flexneri* could survive and enter the VBNC state under nutritional and osmotic stresses at low temperature (4°C). In this state, although the bacteria are alive, they cannot be detected by conventional culture-dependent methods. The infectious dose of the enteropathogen *S. flexneri* is very low. The entry of *S. flexneri* into the VBNC state, coupled with the expression of the *ipaD* and *ipaH* genes and the presence of the virulence plasmid *pINV*, suggests that the bacteria retain their pathogenicity in this state. Furthermore, if the bacteria resuscitate from the VBNC state, it poses a potential risk to food safety and public health.

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### Footnotes

**Authors' Contribution:** Study concept and design: A. N. B.; acquisition of data: A. N. B.; analysis and interpretation of data: A. N. B and M. R. A and E. R. T.; drafting of the manuscript: A. N. B.; critical revision of the manuscript for important intellectual content: A. N. B and M. R. A., and E. R. T.; statistical analysis: A. N. B and M. R. A and E. R. T.; All authors read, revised, and approved the final manuscript.

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