5.1. INTRODUCTION

*Salmonella* is frequently isolated from variety of sources like water, sediments, food and stool samples, which served as a reservoir of this pathogen. *Salmonella* is also persistently released from infected humans, farm animals, pets and wildlife to the environment. In environment, often it experience most critical factors like temperature, pH, salinity, light, radiation, water availability and nutrient deprivation for survival. These adverse environmental conditions are known to induce several bacteria to the VBNC state. In 1982, Xu *et al.* first reported on VBNC bacteria with *V. cholerae* and *E. coli*. Recent findings revealed that many pathogens could enter VBNC state, including more than 67 different species of bacteria including a large number of diarrhoeal causing human pathogens found enters into VBNC state (Oliver, 2010; Ramamurthy *et al.*, 2014 and Senoh *et al.*, 2014). Thus *Salmonella* could also be induced into the VBNC state by adverse environmental conditions (Oliver *et al.*, 1991).

Also, in food processing industry, *Salmonella* encounter various stress conditions, leads to inability to grow in routine bacteriological media and this could be challenging in the food industry. During the unfavourable condition of the bacteria, certain portion of the bacterial population seems to “disappear” in natural water bodies during certain period of the year. It is believed that such disappearance is not due to death, but to their entry into VBNC state, where the cells maintain its cellular integrity and cellular viability, such as respiratory activity, enzyme activity and gene expression [DNA and RNA (Ribo Nucleic Acid)] (Kell *et al.*, 1998; Costa *et al.*, 1999; Signoretto *et al.*, 2000; Oliver, 2005; Anuchin *et al.*, 2009; Cunningham *et al.*, 2009 and Lai *et al.*, 2009). Meanwhile, some cells in this state undergo some morphological transition from rod to coccoid, club shape, budding nature *etc.* (Rahman *et al.*, 1994; Thomas *et al.*, 2002; Adams *et al.*, 2003; Inglis and Sagripanti, 2006; Cook and Bolster, 2007; Du *et al.*, 2007 and Senoh *et al.*, 2010). When the bacteria encounter the nutrient starvation condition, the bacteria, metabolized carbohydrates first, followed by proteins, some
RNA, while the DNA is generally protected (but not always) (Shimizu, 2013). The DNA can also become compressed and surrounded by dense cytoplasm. Unsaturated fatty acid profiles were also found to be increased during VBNC state, probably to maintain membrane fluidity during temperature downshift or modified osmotic concentration (Linder and Oliver, 1989; Day and Oliver, 1998 and Denich et al., 2003). In that stage, the cells cannot longer be recovered/isolated in routine bacteriological media (Kell et al., 1998).

Total cell count which do not indicate actual cell viability but simply the presence of cell. In viability assay, demonstration of VBNC cell is a critical factor. By targeting at different viability indicator, several methods were developed for detection of the VBNC bacteria by direct viable count by using different dye such as 5-cyano-2,3 ditolyl tetrazolium (CTC), SYTO 9 and propidium iodide, 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) to INT-formazan resulting in red spots, 4,6-di-amidino-2-phenylindole (DAPI) direct count DAPI, acridine orange and the BacLight Live/Dead assay kit (Hobbie et al., 1977; Zimmerman et al., 1978; Porter and Freig, 1980; Rodriguez et al., 1992; Coallier et al., 1993; Oliver, 1993; Feipeing and McFeters, 1994; Pyle et al., 1995 and Creach et al., 2003). However, these methods are not capable of analysing environmental samples because of the interference from auto-fluorescence or light-quenching particles in the samples (Liu et al., 2008). Moreover, it is difficult to distinguish specific bacteria from microbial flora in environmental specimens. Some reports suggested that the possibility to quantify VBNC bacterial pathogens in biosolids by quantitative PCR (qPCR) assay (Higgins et al., 2007; Viau and Peccia, 2009). However, a widespread concern is that methods targeted at DNA would lead to a spurious evaluation for the reason that both the live and dead cells could be extracted and detected (Rudi et al., 2005 and Nocker et al., 2006).

The rapidity of the VBNC response to stresses often depends on the physiological age. But, some researchers have argued against the existence of the VBNC terminology. As well as, few studies which often compelling evidence for the VBNC state of certain bacteria like E.coli in filter sterilized lake water, Campylobacter jejuni in 4ºC in sterile sea water, Aeromonas hydrophila cultured in a 0.35% NaCl
solution at pH 7.5 and at 25°C for 50 days, *V. harveyi* SF1 entered into the VBNC state upon incubation in seawater at 4°C for 60 days and *Shigella dysenteria* after uptake of methionine (Rollins and Colwell, 1986; Rahman *et al*., 1994; Porter *et al*., 1995; Rahman *et al*., 2001 and Jia *et al*., 2013). In 1984, Roszak *et al*. first demonstrated that the entry of a *S. Enteritidis* into the VBNC state. Subsequently, various findings supported the concept and it was found that some *Salmonella* serovars such as *S. Typhimurium* (Jimenez *et al*., 1989), *Salmonella enterica* subspecies *salamae* (Monfort, 1994) and *S. Typhi* (Cho and Kim, 1999) enter the VBNC state under different starvation condition. The above reports suggested that the VBNC cells of pathogenic bacteria, especially *S. Typhi* in environment may be the potential etiologic agents. Therefore, the demonstration of a VBNC state of different serovars including *S. Typhi* and *S. Typhimurium* in environment is important for the public health.

Hence, the present study was undertaken to investigate survival strategy of different *Salmonella enterica* serovars like Typhimurium and Typhi (clinical isolates) and *S. Infantis* and Agona (food isolates), analyse the factor which induce the *Salmonella* serovars that are known to enter the VBNC state and also to evaluate the favourable environmental condition to resuscitate back to the actively growing state.

5.2. MATERIALS AND METHODS

5.2.1. Bacterial strain and culture condition

A MDR *S. Typhi* and *S. Typhimurium* strain isolated from patients admitted to government K.A.P. Viswanatham Medical College and Hospital, Tiruchirappalli, Tamil Nadu and MDR *S. Kentucky* and *S. Infantis* isolated from poultry sample were used in this study. The strains were grown for 18 h at 37°C in NA stab and stored at room temperature (RT) for further use.

5.2.2. Culture preparation and inoculation of strains of different *Salmonella* serovars in various starvation media

The NA stab maintained *Salmonella* strains were streaked onto the XLD plates and incubated at 37°C for overnight. After incubation, a single colony was transferred and streaked onto NA plates and incubated at 37°C for 24 h. Well developed and isolated colonies were selected and transferred to LB broth. Cells were grown for
overnight at 37°C O.D.600 of 0.90-1.00 and harvested by centrifugation at 6000 rpm for 5 min. Cells were sequentially washed three times in sterile physiological saline at room temperature to eliminate carryover of any nutrients. The pellets were then finally suspended uniformly in 1:100 of sterile four starvation medium such as low nutrient media [Phosphate buffered saline (PBS), Sea water (SW), River water (RW)) and high nutrient medium (Lactose Broth Difco Ltd-USA)] to give a final concentration of approximately $10^8$ CFU/ml. Finally, all the inoculated culture flasks were then incubated separately in different temperatures like -20°C, 4°C, RT and 37°C.

5.2.3. Investigation of VBNC state
5.2.3.1. Cell enumeration

Aliquots of cell population were aspirated everyday up to five days and periodically at regular intervals thereafter from the starved flask and the cell culturability from each flask was checked in triplicate dilutions in PBS. 0.1 ml of cells was spread onto the NA and XLD plates. All the plates were incubated for 12 to 24 h at 37°C and the colonies were counted. Direct sample (undiluted) had taken from the starvation media, when if the diluted starvation medium did not possess the culturability in both NA and XLD.

5.2.3.2. Live/dead cell count

Viability of the cells were determined by using direct viable counts with Acridine Orange-Ethidium Bromide (AO/EB) staining method, when culturable cells not detected both in selective and non-selective media. For stock preparation, 100 µg of Acridine Orange and 100 µg of Ethidium Bromide were added in individual tube containing 1 ml of PBS. For viable cell count method, 25 µl of cell suspension was mixed with 1 µl of AO/EB solution. Each sample was mixed gently just prior to microscopic observation. Samples were examined immediately. 10 µl of cell suspension was carefully placed onto a microscopic slide, covered with a glass coverslip and examined under fluorescence microscope.

5.2.4. Resuscitation studies

The attempt was made to recover culturable cells from the non culturable (VBNC) state by different resuscitation techniques such as a temperature upshift and
LB medium enriched with 0.1% sodium pyruvate and tween 80 separately were examined as described below.

5.2.4.1. Temperature upshift

A study by Wai (1996) provided evidence for the resuscitation of *Salmonella enterica* cells from the VBNC state following heat shock treatment. Employing their protocol, 10 ml of cells were drawn at each sample points were exposed to various temperatures (20°C to 40°C) at different time period for 15s to 2 days. These heat-shocked samples were plated onto NA plate and incubated at 37°C for 24 h.

5.2.4.2. Reactive Oxygen Species (ROS) scavengers Activity

An attempt has been made following the method of Kong *et al.* (2004) and Oliver (2005). Briefly, 0.1% solution of sodium pyruvate (Sigma-USA) and 0.1% of Tween 80 was directly spread over TSA as well as XLD plates, which were then dried prior to use. All stock solutions were made with filtered, autoclaved, deionized water and kept in the dark at 58°C. At each time point, 100 µl of the starved cells spread onto the ROS scavenger-treated TSA and XLD plates and it incubated at 25°C for up to 48 h.

For the confirmation of the resuscitation, liquid resuscitation medium (0.1% sodium pyruvate) was used. In which 100 µl of the starved cells was incubated with equal volume of liquid resuscitation medium at 25°C for up to 8 days. Based on the turbidity through spectrophotometer analysis, the culturability was monitored every 24 h by spreading starved cells on to the TSA and XLD agar plate.

5.2.5. Cell morphological changes under Scanning Electron Microscopy (SEM)

Morphological changes of the *Salmonella enterica* cells were examined during the stress condition, in dormant state and after resuscitation by SEM analysis. Normal and VBNC cells were harvested by centrifugation at 10,000 rpm for 10 min at 4°C, washed three times with 5 ml of sterile PBS and collected the pellet by centrifugation. Then the cells were freeze dried for microscopic analysis.

The final cell pellet was suspended in approximately 0.1 ml of double-distilled water. One drop was placed on plastic-coated electron microscope grids and allowed to
Samples were shadowed at a 45° angle with chromium and viewed with a high resolution cold Field Emission Scanning Electron Microscopy (FESEM) and its measurement were noted (JEOL, JSM-6701F).

5.3. RESULTS

5.3.1. Culturable count

The survival pattern of human pathogenic different Salmonella serovars (S. Typhi, S. Typhimurium, S. infantis and S. Kentucky) inoculated in different starvation media and incubated in different temperatures are depicted in Fig 22, 23, 24 and 25. All the cells were initially culturable in high number upto 7 days or so and thereafter the counts were drastically decreased.

5.3.2. Survival of Salmonella serovars in Sea water

The survival pattern of various serovars of Salmonella cells were incubated in SW at different temperatures like -20°C, 4°C, RT and 37°C are depicted in Fig 22. The cells of S. Typhi isolated from clinical samples entered into the non-culturable (NC) state after 60 days of incubation at -20°C. However, at the other tested temperature the cells did not enter into the VBNC state and survived up to 300 days. On the other hand, S. Typhimurium at various temperatures entered to the VBNC state after 68th day and cells were not recovered both in NA and XLD plate.

In food isolates, S. infantis entered to VBNC state after 19th day at -20°C, RT and 37°C. But at 4°C the cells did not enter to the VBNC state and survived up to 300 days. In S. Kentucky however, sudden drop in their culturability after 12th day in XLD and NA plate at -20°C and 37°C. At 37°C the cells entered to VBNC state after 47th day. The cells in RT and 4°C did not enter to VBNC state survived better up to 300 days.

5.3.3. Survival of Salmonella serovars in River water

Survival of different Salmonella serovars isolated from clinical and food samples in RW samples at varying temperatures are given in Fig 23. Clinical isolate of S. Typhi and S. Typhimurium incubated separately in RW at -20°C showed a sudden decline in their culturable cells from 10^8 to 0 CFU/ml on 28th day both in NA and XLD.
Figure 22. Survival pattern of Salmonella serovars in Sea water (SW) at -20°C (●), 4°C (■), RT (▲) and 37ºC (▼).
Figure 23. Survival pattern of *Salmonella* serovars in River water (RW) at -20°C (●), 4°C (■), RT (▲) and 37°C (▼).
But, the cells in other temperature did not lose their culturability up to 300 days. In food isolates, S. Infantis at -20ºC, RT and 37ºC lost their culturability after 28th day. Whereas, the cells did not lost their culturability up to 300 days at 4ºC. In S. Kentucky, among the different temperature, it was found that only at -20ºC the cells were influenced and lost their culturability after 28th day.

5.3.4. **Survival of Salmonella serovars in Phosphate buffered saline (PBS)**

Survival strategies of different *Salmonella* serovars in PBS at various temperatures are presented in Fig 24. All the serovars of *Salmonella* were survived better in PBS than other starvation media in all the temperatures tested except at -20ºC. At -20ºC, all the tested strains were gradually reduced their growth and entered to the non culturable state within 20-33 days. In which, S. Infantis have some fluctuation in their culturable count on both NA and XLD plate. Whereas, in other temperature the cells were regularly decreased its growth from $10^8$ to 0 CFU/ml after 300 days.

5.3.5. **Survival of Salmonella serovars in Lactose broth**

The cells of *Salmonella* serovars incubated separately in Lactose broth showed gradual decreases their cell count from $10^8$ to 0 CFU/ml only after 300 days at various temperatures. In which, S. Typhi and S. Typhimurium from clinical samples and S. Infantis from poultry sample had some fluctuation in their growth on both NA and XLD plate at -20ºC (Fig 25). But clinical isolates has some growth fluctuation at -20ºC (Fig 26). Both S. Typhi and S. Typhimurium in NA gradually reduced their growth from $10^8$ to $10^6$ up to 12 days, thereafter it suddenly increased to $10^7$ CFU/ml in 19th day. Till it reached to non culturable state, the fluctuation was continued. In XLD plate, S. Typhi suddenly reduced their growth from $10^8$-$10^3$ within 5th day, from there it again increases its growth rate from $10^3$-$10^8$ in 19th day. Growth variability was observed until it enters to the VBNC state. Whereas, S. Typhimurium in XLD was also reduced their growth from $10^8$-$10^3$ on 40th day. Then it increases their growth to $10^5$ in 47th day. After that the growth was constantly reduced to 0 at 300 days. S. Infantis growth in XLD agar plate also having growth fluctuation throughout the study period. S. Kentucky at 37ºC shows their growth instability in XLD agar plate.
Figure 24. Survival pattern of *Salmonella* serovars in PBS at -20°C (●), 4°C (■), RT (▲) and 37°C (▼).
Figure 25. Survival pattern of *Salmonella* serovars in Lactose broth at -20°C (●), 4°C (■), RT (▲) and 37ºC (▼).
5.3.6. Live/dead cell analysis

The viability of cells of *Salmonella* before and after induction to VBNC state are analysed and presented in Fig 26A and B. It was found that exposure of cells at low temperature (-20°C, 4°C and RT) in low nutrient medium (River water, Sea water and PBS) resulted in decrease in culturability and further leads to non culturability and cell death. The dramatic loss in culturability in both starvation medium led the study to test the presence of the viable bacteria despite its inability to grow on laboratory media through membrane integrity or metabolic activity under microscopic observation. Fig 26B showed that approximately half of the cells were green that emitted green fluorescence, indicating these cells had an intact cellular membrane which is indicative of live cells. The red fluorescing cells lacked cell membrane integrity and represent dead cells. Hence, the study suggested that a significant number of the inoculum was maintaining membrane integrity, which indicated that the cells are viable and not dead. This proved the concept of VBNC.

![Figure 26. Fluorescent microscope displays Live/ Dead cells. *Salmonella* strain stained with Acridine orange/Ethidium bromide. Figure 26A only Live cells shown Figure 26B it shows Live/ Dead cells with green and orange colour.](image)

5.3.7. Resuscitation from the non culturable state

In the present study, the attempt was made to analyse the recovery of non culturable *Salmonella* serovars by using two different types of techniques such as temperature upshift and addition of ROS scavengers (Sodium pyruvate and Tween 80). In temperature upshift experiment, various temperature ranges were used to resuscitate the non culturable cells and are presented in Table 10. In this technique, non culturable
cells of *S. Typhimurium* was successfully resuscitated after 2 h 30 min at 37°C, whereas in other low time period the cells were not able to grow (Fig 27). Resuscitation attempt was also made in the presence and absence of sodium pyruvate and Tween 80. 0.1% of sodium pyruvate (solid and liquid) induced the cells to resuscitate from the VBNC nature to culturable state (Fig 28) particularly cells from RT and 37°C. Addition of Tween 80 did not support the growth of VBNC cells. This might be the toxin deposition and this is one of the factors to influence the *Salmonella* to enter the VBNC state.

![Figure 27](image1.png)

**Figure 27.** Comparison of nonculturable *S. Typhimurium* exposed to an 35°C upshift for 2 h (right-B) versus no temperature upshift (left-A) followed by inoculation on TSA at 37°C for 24 h.

![Figure 28](image2.png)

**Figure 28.** *Salmonella* resuscitation by the addition of 0.1% sodium pyruvate. Figure 28A *Salmonella enterica* growth in solid XLD medium containing 0.1% sodium pyruvate, Figure 28B Turbidity was observed after resuscitation liquid broth containing 0.1% sodium pyruvate and Figure 28C represents that the resuscitated *Salmonella enterica* cells on XLD from 0.1% sodium pyruvate containing liquid medium.
Table 10. Resuscitation of \( S. \) Typhimurium cells from non culturable state by temperature upshift method

<table>
<thead>
<tr>
<th>Temperature upshift</th>
<th>No dilution</th>
<th>( 10^{-1} )</th>
<th>( 10^{-2} )</th>
<th>( 10^{-3} )</th>
<th>Cfu/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-10 min</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10-30 min</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>30-60 min</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>60-120 min</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>At 150 min</td>
<td>18</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>18</td>
</tr>
</tbody>
</table>

5.3.8. Morphology of \( S. \)almonella in log and starved phase

The cells of different serovars of \( S. \)almonella exhibited morphological changes, during the survival strategy experiment through SEM analysis are presented in Fig 29. Fig 29A showed that representative microscopic fields of laboratory grown young cells which were typical rod shape. After 30 days of incubation at -20°C in sea water, it was noticed that the cells of \( S. \) Kentucky entered to VBNC state and their structure was not intact (Fig 29B). But, during the non culturable state morphological changes in the \( S. \) Typhimurium cells were transformed from rod to coccoid shape and some cells were in the destructive nature (Fig 29C). After resuscitation of the \( S. \) Typhimurium, some cells were in elongated in nature and most of the cells were short rod in shape (Fig 29D). A change in morphology indicates that the \( S. \)almonella lost its cell permeability during dormant state.
5.4. DISCUSSION

The VBNC form of cell is a metabolically active one, while cells are incapable of undergoing the constant cellular division essential to form a colony on regular bacteriological media. The existence of the VBNC state was first reported by Dawe and Penrose in the year of 1978, who presented evidences that coliforms when present in sea water, did not die off for long periods. On the other hand, Stevenson (1978) speculated that the small bacterial cells routinely observed by direct microscopy in sea water might be in a dormant state resulting from starvation and the cells may be in a non-culturable state. However, first it was scientifically discovered in 1982 and reported that *E. coli* and *V. cholerae* cells could enter a distinct state called the VBNC state (Xu *et al.*, 1982). More than 61 species contained in 32 genera has been demonstrated as VBNC state till now (Oliver, 2005).
The present investigation was focused on survival of various *Salmonella* serovars in different starvation media incubated at different temperature like -20°C, 4°C, RT and 37°C. Results of this study indicated that the culture of bacteria on standard artificial media under laboratory conditions is fundamentally different from the conditions met in a natural environment. The optimal survival of *Salmonella* serovars was observed in 4°C and RT in low nutrient medium and all the tested temperature in high nutrient medium. This result was supported by Pavic (2010), who inoculated the *Salmonella* culture into egg and incubated at different temperature and the faster penetration was observed at 4°C and 25°C. The ability of this bacterium to persist or grow at ≤10°C is of more significance. In 1989, however, Humphrey *et al.* reported that *S. Typhimurium* did not grow at 4°C and 8°C. The results suggested that the longer survival of salmonellae at lower temperatures was due to a slower metabolic rate, induced by adverse conditions. In the present study, conversion to VBNC state cells were detected, when the cells of *Salmonella* were incubated in low nutrient medium at -20°C and 37°C when incubated upto 200 days. Among the different starvation medium, sea water found to mainly influences the VBNC state of the *Salmonella*. This was strongly in agreement with the work reported elsewhere (Roszak and Colwell, 1987; Oliver *et al.*, 1991; McKay, 1992; Chaiyanan *et al.*, 2001; Besnard *et al.*, 2002; Amel *et al.*, 2008 and Davidson *et al.*, 2015) and this may because of insufficient nutrients in low nutrient medium to support the growth. The duration for entry into VBNC state by *Salmonella* simply rely on nutrients for their survival wherein the available nutrients might be fully exploited by the number of cells in the medium or salinity may also contributed the VBNC state of *Salmonella* in the natural aquatic environment. This abnormal salinity might become a stress factor thereby making it to enter into dormant state within a shorter duration (Marco-Noales *et al.*, 1999).

Temperature is also found the major factor to induce VBNC state of *Salmonella* when incubated at -20°C as well as 37°C. Similar results were reported in *Pseudomonas* spp., *E. coli*, *Listeria monocytogenes*, *Salmonella enterica* and other bacterial species by Oliver *et al.* (1995), Wang and Doyle (1998); Besnard *et al.* (2002) and Zeng *et al.* (2012). This could be due to the extreme lowering temperature would decrease the fluidity and permeability of the lipids in the phospholipid bilayer of the outer membrane (Vandemark and Batzing, 1987). Meanwhile, range of salinity at different
temperature also significantly affects the survival ability of the bacteria. This might be influence the *Salmonella* enter to the dormant state during the study period. This was already reported by Marco-Noales *et al.* (1999). Also the effect of temperature and osmotic pressure appears to be extremely important starvation will alter membrane composition (Morishige *et al*., 2014). Oliver *et al.* (1991) also reported that the VBNC state is typically induced efficiently in many bacteria by a combination of environmental stresses and often by starving cells in a nutrient-limited medium while simultaneously incubating them at a low temperature.

In the present investigation it was found that the plate count of *Salmonella* serovars varied from day one to several days to enter VBNC state, which clearly indicated that, the entry of VBNC state not only in days dependent but also influenced by some other environmental stress condition (low temperature, high osmolality and nutrient starvation) within the serovars of *Salmonella*. However, in environmental strain like *S.* Kentucky and *S.* Infantis suggesting lost their permeability in very short period of time and entered into dormant state in low nutrient medium in order to maintain its virulent potential. This was supported by Morishige *et al.* (2014) who found similar kind of results in their study. Zeng *et al.* (2012) was observed that *S.* Typhi entered into VBNC state within 12-14 days, while Smith *et al.* (1994) noted variation in the entry of VBNC state by *S.* Typhimurium from 54 to 56 days. The present data are completely in agreement with the above works and this may because of considerable variation in the cellular composition of the different serovars of the *Salmonella* cells or variation in the initial inoculum size.

Few studies were demonstrated that plate counts were dramatically underestimated the total number of bacteria present in starvation media. Hence, in the present study, an attempt was made to view the viable cells present in starvation media and the known samples from all the media were subjected to AO/EB staining. The above technique was followed by many workers and some other techniques were also used to identify the total viable nature of the cells present in the sample (Kogure *et al.*, 1979; Roszak and Colwell, 1987; Barcina *et al*., 1995; Boulos *et al*., 1999; Wong and Wang, 2004 and Pawlowski *et al*., 2011). Most of these studies were used Backlight kit method, which were equal to those obtained with acridine orange staining method.
Acridine orange is a vital dye, interact with nucleotides, whereas ethidium bromide intercalate inside the DNA double helix and will stain both live and dead cells. Ethidium bromide will stain only cells that have lost membrane integrity, whereas dead cells have a damaged membrane that is unable to retain chromosomal and plasmid DNA, while VBNC cells have an intact membrane containing undamaged genetic information (Heidelberg et al., 1997; Kasibhatla et al., 2006 and Cook and Bolster, 2007). This may because during starvation condition the bacteria had changes its membrane integrity or having small amount of nucleic acid.

Some studies are successfully resuscitating the culturable cells of *Salmonella* from non culturable state by using temperature upshift method (Oliver et al., 1991; Wolf and Oliver, 1992; Oliver and Bockian, 1995 and Gupte et al., 2003). In the present study, resuscitation of stressed bacterial cells was attempted by nutrient medium enriched with sodium pyruvate and temperature upshift method. Among the various serovars of *Salmonella* tested, *S. Infantis*, *S. Kentucky* and *S. Typhimurium* cells lost its culturability but not viability at different temperature: at the same time they retain its significant level of culturability after nutrient supplement and in different temperature upshift method. The recovery of *Salmonella* was found high in liquid medium compared with solid medium supplemented with sodium pyruvate. Hence, sodium pyruvate able to promote significant resuscitation of populations by removing toxic material from the cells that was so heavily stressed than the other supplements. Temperature may also induce the main stress to this *S. Typhimurium*, because when temperature increased from -20°C to 35°C, the stress may remove from the cells, thus allowing the cells continued participation in the overall ecology of the environment. Thus, the appearance of culturable cells following resuscitation must have been due to true resuscitation of the nonculturable population.

An alteration in cell morphology due to starvation stress was examined by SEM. The control *Salmonella* cells, had a normal rod shape with a smooth surface. During VBNC period, some cells were found to change its morphology and some cells were in the form of destructive nature. These changes were observed in several bacterial species including *Salmonella* serovars were reported by many investigators (Kjelleberg et al., 1993; Morita, 1993 and Jiang and Chai, 1996). In 2004, Kalchayan
et al. also observed this destructive morphology in *Salmonella* in VBNC state. Whereas, in the present investigation the cells obtained through resuscitation at 35°C, the cells continued to metabolise nutrients and became elongated and flattened after 24 h incubation. The size of metabolically active cells increases, while inactive cells remain unchanged as measured by SEM analysis (Fig 30D).

Variety of studies has shown that introduction of *Salmonella* in VBNC state was influenced by low temperature, oxidative stress condition, various osmotic condition, different nutrient availability and radiation (Oliver, 1993; Cho and Kim, 1999; Gupte *et al*., 2003; Lindback *et al*., 2010 and Morishige *et al*., 2014). However, from the present study it was suggested that temperature alone may not induce the VBNC state of *Salmonella* in nutrient poor environmental conditions and also a combination of nutrients and ambient temperatures might have triggered increasing the population of *Salmonella*. The fact that during the non culturable state, these pathogens can persist in the environment and produce toxin also increase its virulence and resistance to stress and antibiotic agents. Hence, eradication of these VBNC cells need prolonged antibiotic treatment and this state is a true public health concern. It is concluded from this study that interactions between various environmental stresses on the induction of the VBNC state is more complex phenomenon. During VBNC state, the pathogenic bacteria maintain their pathogenic genes and/or factors. This state to be consequence, the cells are capable of resuscitate in some environmental stimulus. It became metabolically active and culturable and are capable of resuscitation under favourable conditions, thus pose a major potential health risk. As such, VBNC state of pathogenic bacteria in food is potentially harmful to public health and should be considered at the time of food inspection. From the public health point of view, it is ascertain to know the potential risk resulting from climatic changes in the natural ecosystems which in turn change in various environmental parameter including the induction of such virulent bacteria into non-culturable dormant state and recovery of inhibited virulent characters be made possible after subsequent resuscitation. While resuscitation is a complex process, during which many reactions and pathways must be switched on in a temporally controlled and coordinated manner. On the other hand, a true physiological response will depend on the identification and characterization of the genetic determinant that regulate this response, which warranting on extensive study.