Bacteriology (CC - 2)

Bacteriological Techniques (Unit - 2)

Accessing Nonculturable Bacteria

Viable but Nonculturable Bacteria (VBNC)

- VBNC bacteria cannot be cultured on routine microbiological media, but they remain viable and retain virulence
- The cells that form a colony on specific nutrient media are the culturable cells.
- Viable means metabolically or physiologically active. So the cells that are metabolically or physiologically active but cannot be cultured on specific media are the viable but nonculturable cells (VBNC)
- In 1982, Xu and co-workers introduced the term “viable but nonculturable bacterial cells (VBNC)”
- Microorganisms that do not grow in culture media, but are still metabolically active and capable of causing infections in animals and plants, are said to be in a VBNC state

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- Traditional laboratory culture conditions and methods cannot meet the requirements of VBNC organisms to resume growth
- They have been detected by observing discrepancies between plate count enumeration of bacterial population and direct staining and microscopic counts
- The viable but nonculturable (VBNC) state is a unique survival strategy of many bacteria in the environment in response to adverse environmental conditions

- The VBNC state is defined as a state of dormancy triggered by harsh environmental conditions, such as nutrient starvation, extreme temperatures, and sharp changes in pH or salinity; osmotic stress, oxygen availability, and damage to or lack of an essential cellular component including DNA; exposure to food preservatives and heavy metals; exposure to white light; activation of lysogenic phages or suicide genes such as *sok/ hak* or autolysins; and decontaminating processes such as pasteurization of milk and chlorination of wastewater
- A good number of bacteria including many human pathogens have been reported to enter the VBNC state
- Most of the bacteria that enter VBNC state are gram-negative species belonging to the gamma subclass of the proteobacteria branch

- The following list includes but is not limited to the pathogenic bacteria that can enter the VBNC state: *Agrobacterium tumefaciens, Campylobacter jejuni, Enterobacter aerogenes, Enterococcus faecalis, Escherichia coli (including EHEC), Helicobacter pylori, Klebsiella pneumoniae, Legionella pneumophila, Listeria monocytogenes, Mycobacterium tuberculosis, Pseudomonas aeruginosa, Salmonella typhi, Salmonella typhimurium, Shigella dysenteriae, Shigella flexneri, Shigella sonnei, Streptococcus faecalis, Vibrio cholerae, Vibrio parahaemolyticus* etc.

- VBNC pathogenic bacteria are considered a threat to public health and food safety due to their nondetectability through conventional food and water testing methods.
- A number of disease outbreaks have been reported where VBNC bacteria have been implicated as the causative agent.
- Further molecular and combinatorial research is needed to tackle the threat posed by VBNC bacteria with regard to public health and food safety

Methods of Detection of VBNC Bacteria

- **Bright Field Microscopy with Nalidixic Acid*:***

  Nalidixic acid (20–40 mg/L) is used to stop cell division. After exposure to nalidixic acid, viable cells continue to grow and will appear elongated, whereas the nonviable metabolically inactive cells will retain their original shape and size. The cells are then observed under a microscope. Viable cells will be seen as elongated, whereas VBNC/dormant cells will be seen as oval and large

- **Fluorescent Microscopy*:***

  Various fluorescent staining procedures can be used to determine VBNC organisms. Frequently used stains are acridine orange, 4,6-diamino-2-phenyl indole (DAPI), fluorescein isothiocyanate (FITC), indophenyl nitrophenyl-phenyltetrazolium chloride (INT), and 5-cyano-2,3-ditolyl tetrazolium chloride (CTC)
- The mode of action of these dyes and the reactions observed are summarised in the following Table

Dye	Mechanism	Reaction
Acridine orange	The staining response depends on the ratio of DNA to protein in the cells	Actively reproducing cells appear green but slow-growing or nonreproducing cells at time of staining appear orange
Di-amino-phenyl-indole (DAPI)	Differential staining	Living cells look green under fluorescent microscope
Indophenyl nitrophenyl -phenyl tetrazolium chloride (INT)	INT reacts with dehydrogenase enzyme to produce formazone and red colour, thus living cells appear red	Living cells appear red
Fluorescein isothiocyanate (FITC)	Enzyme activity in living cell	FITC stains living cells violet or blue

- In recent years, a new **differential staining assay**, the **BacLight Live/Dead assay**, has been developed.
- The assay allows simultaneous counting of total and viable (metabolically active) cells, by using two nucleic acid stains, that is, greenfluorescent SYTO 9 stain and red-fluorescent propidium iodide stain.
- SYTO 9 stains both live and dead bacteria, whereas propidium iodide penetrates only bacteria having damaged membranes.
- When used together, propidium iodide reduces SYTO 9 fluorescence in dead bacteria with damaged membranes resulting in red fluorescent cells, whereas the live bacteria will fluoresce green

- **Molecular Techniques*:***

  Hybridization probes are nucleic acids (DNA/RNA) which have been chemically or radioactively labelled and are used to detect complementary target DNA/RNA
- Due to the failure of distinguishing between dead or live cells by DNA-based methods, RNA-based methods are a more valuable estimate of gene expression and/or cell viability under different conditions. This technique is more able to discriminate between culturable and nonculturable forms of an organism
- Specific amplification of RNA targets permits detection of specific organisms or groups of related organisms without the need to cultivate them, provided the appropriate unique primers are used

- **Reverse transcriptase PCR** (RT-PCR) can distinguish between live and dead cells. This is possible because it is an mRNA-based method and mRNA is short-lived (half life less than 1 minute).
- Messenger RNA is only present in metabolically active cells and not found in nature after cell death.
- RT PCR can detect nonculturable but active or live cells

- Oligonucleotide probes of l8–20 nucleotides are proving most useful because they hybridize rapidly to specific DNA sequences of target organisms. These gene probes can reveal closely related organisms or organisms with similar functional capabilities
- The detection of VBNC cells directly from the environmental samples can also be achieved using different types of blotting such as colony blot, slot blot, dot blot, and southern blot. The principle of blotting is the use of radio- or nonradioactive or fluorescence labelled probe

- **Fluorescent in situ Hybridization** (FISH) is an alternative format for hybridization probes in which fluorescence labelled DNA or RNA probes are hybridized with target nucleic acids in whole, permeabilized cells.
- The application of this method to the detection of single microbial cells by using rRNA-targeted probes in combination with epifluorescent microscopy has been developed
- This is done through selective targeting of regions of rRNA, which consist of conserved and variable nucleotide regions.
- By choosing the appropriate rRNA probe sequence, FISH can be used to detect all bacterial cells (a universal probe) or a single population of cells (a strain-specific probe) of VBNC.
- It has lower sensitivity and cannot distinguish live and dead cells

- Even though traditional culture methods fail to detect the presence of specific VBNC in a sample, the presence of these microbes can be demonstrated using some of the molecular techniques described