

## Viable but non-culturable bacteria: their impact on public health

More than 50 years ago, standard microbiological methods were developed to determine whether bacterial cells were dead or alive. This is an important question because the answer is the basis of decisions such as safety of food and drinking water, sterility of pharmaceuticals, recurrence of infectivity and spoilage of treated industrial products. The viability of a bacterial cell was traditionally determined by its ability to grow and produce colonies. However, in recent years, many studies have revealed the ability of both Gram-positive and Gram-negative bacteria to go into a viable but non-culturable (VBNC) state. In this state, bacteria are still viable and show metabolic activity and respiration, but cannot be shown as colony forming units by the conventional plate counts and hence remain hidden<sup>1</sup>.

Microbial ecologists have in the past stated that plate counts of bacteria in soil, rivers and oceans are typically less than 1% of the total bacteria observed by direct microscopic methods. This was earlier attributed to seasonal die-off of cells, but is now considered to be due to the entry of bacteria in the VBNC state. Factors affecting non-culturability of bacteria could be lethal/sub-lethal injury of cells, low temperature, adaptation and differentiation, nutrient substances accelerating death, and lysogenic bacteriophages among others<sup>1,2</sup>.

VBNC bacteria are a major concern in public health risk assessments because many pathogenic bacteria like *Vibrio cholerae*, *Mycobacterium tuberculosis*, *Campylobacter jejuni*, *Helicobacter pylori*, *Vibrio vulnificus* and *Escherichia coli* have been reported to enter a VBNC state from which they are able to return to the infectious state after passaging in animal hosts<sup>3-5</sup>. Reports indicate that many potentially harmful bacteria survive treatment and persist in processed food, pasteurized milk, potable water and in the environment<sup>1</sup>.

The VBNC state has been particularly well-studied in case of *V. cholerae*. Scientists were finding it mysteriously difficult to isolate *V. cholerae* from environmental samples throughout the year, even in cholera-endemic countries like Bangladesh<sup>6</sup>. However, discoveries of the past decade have revealed the existence of a dormant VBNC state which *V. cholerae* O1 enters in response to nutrient deprivation and other environmental conditions. Counts of bacteria obtained by microscopy were often 200 to 5000 times more

than the number of colonies on the plate. It is now known that *V. cholerae* is in fact indigenous microflora of the aquatic environment. Resuscitation of VBNC cells has also been reported. It is now known that the adverse nutritional conditions and fluctuations in temperature can cause *Vibrio* to become non-culturable. The cells lose their ability to be cultured in a linear manner, eventually reaching a point where plating suggests a total lack of living cells. The primary evidence that such cells are alive is obtained when one of the direct viability assays is applied to such cultures. These assays allow direct determination of viability of individual cells in a population without the need for culture.

Substrate uptake experiments reveal that VBNC bacteria maintain viability in a state of greatly reduced substrate uptake and metabolic activity. Cells entering VBNC state undergo a reduction in size becoming ovoid and with significant changes in membrane structure, protein composition and ribosomal content. For instance, *V. vulnificus* is known to produce 40 new proteins not seen during growth at normal temperature. Many believe that the VBNC state is the programmed genetic response of a cell to overcome adverse conditions<sup>1,2</sup>.

Various methods have been adapted to enable reliable detection of VBNC bacteria such as epifluorescent microscopy combined with flow cytometry and molecular biology techniques. However, variable persistence of nucleic acids in dead cells can pose a problem. Recently, Pai *et al.*<sup>7</sup> have described a two-tube directed RT-PCR for detection of mRNA of antigen 85B (Ag 85B) of *Mycobacterium tuberculosis*, that can distinguish between viable and non-viable organisms. Ag 85B is abundantly secreted by *M. tuberculosis* and is hyper-expressed under stress conditions: thus the method to identify its mRNA is useful in detecting viable but dormant bacteria<sup>7</sup>. Divol *et al.*<sup>8</sup> have detected non-culturable yeast in botrytis-affected wine using direct epifluorescence, PCR-RFLP and PCR-denaturing gradient gel electrophoresis. Gunasekera *et al.*<sup>9</sup> have assessed viability of bacteria in pasteurized milk using expression of a *gfp* reporter gene and membrane integrity based on propidium iodide exclusion as viability indicators. The combination of a dye that targets the activity of a cellular function with another that preferentially stains dead cells is of great interest. This approach

has been developed in the BacLight LIVE/DEAD staining kit from Molecular Probes Inc and CV3/CSE labelling kit from Chemunex.

The implications of bacteria to lie in a dormant undetected state are far reaching. For instance, antibiotic resistant chronic otitis medium was earlier considered a sterile inflammatory process. But it is now known that there are antibiotic-resistant bacteria in biofilms which are in VBNC state, causing chronic infection<sup>10</sup>. The sudden recurrence of tuberculosis years after a person was presumed cured has also been attributed to resuscitation of VBNC *Mycobacterium* cells<sup>7</sup>.

Although the discovery of VBNC bacteria has highlighted the significance of growth-independent microbial detection procedures, it is a fact that standard culture-based microbiological methods have proven to be sufficiently effective to protect public health for many decades. For developing nations such as ours, the simplicity, cost-effectiveness and reliability of standard methods cannot be undermined. However, in public health risk assessments and epidemiological surveys, standard methods should be supplemented by selected growth-independent assay procedures for strategic monitoring of viable but non-culturable microbes.

In addition to public health, the VBNC state has tremendous implications for terrestrial biogeosciences, astrobiology and policy regarding release of genetically modified organisms.

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**Yogita N. Sardesai**, Goa College of Pharmacy, Panaji, Goa 403 001, India  
e-mail: nnnps@sancharnet.in