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The Viable but Nonculturable Concept, Bacteria in Urine Samples, and Occam's Razor

Discrepancies between the number of bacterial cells that can be detected by a direct counting method such as microscopy and the number that form colonies on standard media are regularly reported and extensively discussed (3). The observations of Anderson and colleagues concerning urine specimens (1) fall into this category and are without doubt of considerable interest and potential importance. The intriguing conclusion that there is an abundant source of nonculturable prokaryotic cells in the urinary tracts of humans and mice seems unavoidable. However, we are concerned that the title of the paper and the assumption that the bacteria detected were in the viable but nonculturable (VBNC) state is not justified by either those authors' data or the preceding literature. In particular, the implication that the cell populations termed VBNC by Anderson et al. are in a specific physiological state distinct from starvation is highly contentious and potentially misleading.

Notwithstanding problems with the term VBNC (2, 4, 9), our principal concern is that undue emphasis has been given to one interpretation of the results of Anderson et al., which refers to a hypothetical bacterial condition at the expense of other interpretations whose physiological basis is extensively validated. Specifically, we dispute the view that excludes the possibility that the populations labeled VBNC were injured, moribund, or even dead. Anderson et al. define viable cells as those fluorescing green with the LIVE/DEAD reagent, and they calculate the size of the VBNC population by determining the difference between the colony count and the green-cell count. They argue that their VBNC populations were "actually viable" on the basis that lethally UV-irradiated cells acquire the "dead" labeling phenotype more quickly than the cells in their samples. However, killing cells by lethal doses of UV does not address the real issue of whether any of the likely processes by which the bacteria in their samples may have been killed could result in a sustained "live" labeling phenotype. Indeed, cells with intact membrane signals have been shown directly to have lost reproductive capacity (6). Most importantly, we and others argue that the term "viable" should be reserved for cells that retain the capacity for reproduction. Andersen et al. make no attempt to address this point and, in doing so, ignore key aspects of the preceding literature. Some attempt should have been made to resuscitate putatively VBNC cells. Moreover, simple tests could have been done to determine the presence of peroxide-sensitive cells (5), injured cells (8), and cells capable of growth only in broth (7).

While numerous studies have demonstrated nonculturable bacterial populations that retain cellular indicators of integrity or activity, very few have demonstrated that any of these cells can return to culturability. In the latter instances, the period during which the return to culturability was demonstrated was brief. Furthermore, it has been argued that such results are due to injured cells on a pathway of unrelenting deterioration to complete loss of culturability (4, 5). Thus, we dispute the view that truly viable but temporarily nonculturable cells can "remain in this condition for long periods."

Our central point is that many established and fully validated descriptors might be applied to the nonculturable cells detected in this study. Occam's razor, which urges us to go with the simplest available explanation that is consistent with the observations at issue, provides a radically different interpretation of Anderson et al.'s data. This simpler explanation is that the observed nonculturable cells are either dead or passing through a brief injured state to death. This interpretation, in our view, renders the VBNC concept redundant.

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Author's Reply

Drs. Barer and Bogosian raise some excellent points in their letter. The term "viable but nonculturable" (VBNC or VNC) is not an accurate description of the bacterial physiological condition it is meant to describe. It is used here because it is the standard term in the field and its meaning is understood to be as follows: viable cells that do not give rise to visible growth under nonselective conditions that normally support growth (for multiple reviews on VBNC cells, see reference 3). This operational definition allows one to differentiate VBNC cells from those that are starved and wounded or injured. This difference is beginning to be documented at the molecular level (5, 8). That VBNC is not a transient cellular response is indicated by the ability of VBNC cells to remain in, and be resuscitated from, this state for long periods of time—in one report, for more than 5 years (7). The term VBNC is inaccurate because "nonculturable" cells are considered to have the potential to grow or resuscitate. Because one assays only a

sample of bacterial cells in a population to determine the physiological status of cells in the population, even the numerous reports of resuscitation can be subject to criticism (2).

Drs. Barer and Bogosian's other issues relate to two limitations currently inherent to the VBNC field: the need to use growth-independent viability assays and the absence of universal resuscitation conditions (which are not always a simple reversal of the induction conditions). The commercial LIVE/DEAD *Bac*Light viability assay (Molecular Probes, Inc.) used in our study (1) assays for intact cell membranes. False-positive results should arise only if cells have not been dead for a time sufficient for their membrane integrity to be compromised. That "reproductive ability can be lost prior to the loss of membrane integrity" was demonstrated by Ericsson et al. (4), who also concluded that the "LIVE/DEAD *Bac*Light assay accurately reports on the viability of the growing and stationary-phase *E. coli* culture analyzed." The question, then, becomes how long it takes for a dead cell to lose its membrane integrity. We addressed this source of false-positive results by directly measuring the time it would take for the membranes of cells killed by a method that does not damage the membrane to become compromised and then examining our samples after a longer period of time.

The term "injured" used by Bogosian et al. (2) seems to be different from the terms "wounded" and "injured" used by McFeters et al. (see reference 6) and "injured" used by Weichart and Kjelleberg (9). The term "injury" seems to imply, as is stated directly for "wounded" and "injured" (6) and "injured" (9) cells, an ability to regain growth ability. If so, then the difference between VBNC and injured cells may be less significant, and it does not detract from determining the biological significance of and from understanding the mechanism behind why there are viable cells in samples where none are thought to be. If cell injury describes an irreversible pathway to death, then it raises some interesting questions. Should cells at the entry to this irreversible pathway be considered "dead"? If not, at what point do they "die," and how, then, is "death" defined?

The growing interest in the VBNC field is based upon this condition being able to explain many laboratory and field phenomena and upon the potential impact of these bacteria on biological phenomena, such as disease etiology. The purpose of the study of Anderson et al. (1) was to document the potential relevance of the VBNC condition to urinary tract infections and to engender additional study in this area. As long as experiments consist mainly of describing phenomena, results will be affected by unknown factors, and there will continue to be valid differences of opinion. The use of molecular tools may help to elucidate the genetics underlying VBNC conditions and resolve the controversy.

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