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OPEN JOURNAL OF Environmental Biology OPEN ACCESS JOURNAL

ISSN: 2690-0777 DOI: https://dx.doi.org/10.17352/ojeb

Short Communication

Viable but non-cultivable bacteria and their implications for microbiological safety

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Received: 12 April, 2022
Accepted: 31 May, 2022
Published: 01 June, 2022

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Communication

Bacteria are microorganisms found in all environments and they fulfill various tasks in ecosystems, however, some can affect the human population causing outbreaks of disease and death. The most common forms of exposure and infection are contaminated food and water. That is the reason there are different organizations in the world that study the incidence of gastrointestinal and/or enteric diseases. For example, in the United States, there is the Active Surveillance Network of Foodborne Diseases (Food Net), which monitors 10 sites for infections that are diagnosed in the laboratory by eight pathogens (*Campylobacter, Cyclospora, Listeria, Salmonella, Shigella, Vibrio, Yersinia* y *Escherichia coli*). Their data, in 2019, revealed 25,866 infections; 6,164 cases requiring hospitalization, and 122 deaths. Without forgetting that, according to the WHO, diarrhea remains the second cause of death in children under 5 years in the world, which translated (in 2019) into the death of 525,000 children globally and 9,904 in Latin America [1].

Trying to reduce these figures, the authorities implement regulations and standards that specify the limits of abundance and methods for the detection of pathogenic microorganisms, which are usually based on cultivation techniques in Petri dishes where the Colony Forming Units are counted. So, if a plate shows a clean culture medium (without colonies), the sample is considered to be free of bacteria and therefore safe. That is, the microbiological safety of a sample is measured by the cultivability of the species present in it.

However, in 1982, Xu, et al. discovered the existence of the Viable but Non-Culturable (VBNC) state. Which was defined as a tactic of non-spore-forming bacteria to survive in front of environmental stressors, which include the most common disinfection methods such as exposure to UV light [2], antibiotics [3], chlorine [4], high or low temperatures [5], high or low pH levels [6], as well as food preservation processes such as salting [7], dehydration [8] or freezing [9], which were considered "safe".

Upon entering into the VBNC state, the cell undergoes certain changes, for example, morphological alterations ranging from size reduction [5], to changes in the composition of the cell wall and membrane, including proteins, fatty acids, and peptidoglycan [10]. Other modifications are the decrease in metabolic rate [11], microorganisms showing greater physical, and chemical resistance [12] and antibiotics, which differ in gene expression, change their adhesion properties [13] and their potential for virulence, since some cannot cause diseases until they recover their cultivable state, although others maintain their pathogenicity by continuing to express toxins [14].

However, VBNC bacteria differ from dead cells since their membranes are not damaged and retain genomically or plasmid DNA, maintain their respiratory and metabolic activity, and even possess high levels of ATP [15], but decrease nutrient transport and macromolecule synthesis [16]. Despite the above, they don't replicate, therefore, they are not quantified by conventional methods such as Most Probable Number (MPN) or Plate Count Agar (PCA), since these techniques are based on counts of bacterial colonies developed on a Petri plate,

Citation: Jiménez-Moleón MC, Solano-Gómez JA (2022) Viable but non-cultivable bacteria and their implications for microbiological safety. Open J Environ Biol 7(1): 014-016. DOI: https://dx.doi.org/10.17352/ojeb.000028

a liquid or semi-solid medium, during incubation, that is, on their cultivability.

In addition, it has been discovered that VBNC bacteria can be resurrected when the environment becomes favorable again. The term "resuscitation" refers to the fact that they recover their ability to cultivate and return their physiological characteristics and metabolic processes to normal. This resuscitation can be reached by stimulating factors such as an increase in nutrient concentration, increase or decrease in temperature, the presence of chemical stimuli, co-culture with host cells [16], quorum sensing autoinducers (cell-cell communication system), active proteins (factors that promote resuscitation Rpfs, YeaZ and catalase) [4]. However, it is important to mention that bacteria in the VBNC state can only be resurrected within a specific period in the so-called "resuscitation window", their duration depends on the species, bacterial age, and the conditions that induced the VBNC state and the resuscitation conditions. If they exceed this window, VBNC cells can still survive for some time, but eventually die [14].

At this time, it is important to remember again that the standard techniques to know the bacteriological quality of the different matrices are based on the count of the Colony Forming Units (CFUs) using solid, liquid, or semi-solid culture media, but since VBNC bacteria are not able to form colonies, these methods do not allow to count the real number of viable bacteria present and underestimate the microbiological contamination. To avoid this serious microbiological safety problem in different matrices, there are viable bacterial detection methods such as [17]: fluorescence microscopy including staining with acridine orange and nalidixic acid or countersanctions with 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) and 4,6-diamino-2-phenylindole chloride (DAPI) or molecular methods such as quantitative polymerase chain reaction (qPCR) combined with monoazide propidium (PMA) or reverse transcriptase-polymerase chain reaction (RT-PCR).

Currently, a large number of bacteria entering the VBNC state and even some species of fungi have been identified [14]. Most bacteria are Gram-negative, although there are also Gram-positive and several of them, are pathogenic [18]. In fact, it is considered that most non-specific food infections may be related to the resuscitation of bacteria that entered the VBNC state during their previous handling.

The existence and significance of VBNC bacteria within the "human system" has been presented, but what about these bacteria in the VBNC state in the environment? In the marine environment, many bacteria have been associated with dormancy, typified as the VBNC state [19]. Bacteria found in the sea are exposed to a natural environment that has various stressors that cause the induction of the VBNC state [20,21]. As in other matrices, later, when they come into contact with stimuli such as increased temperature, they resurrect.

An example of its importance is the bacteria of the Vibrio genus that are found in estuarine and brackish surface waters, this pathogen is the cause of diseases in animals and humans such as infections, diarrhea, cholera, gastroenteritis, and septicemia [20,22]. People can come into contact with these microorganisms through food, such as eating oysters, which are products that are usually consumed raw or undercooked. When these mollusks are contaminated with *Vibrio vulnificus*, they cause infections such as gastroenteritis with abdominal pain, diarrhea, and vomiting, which can progress to primary septicemia. It has been reported that these bacteria enter the VBNC state due to exposure to low temperatures (during the winter months) and that they can resuscitate both when the season changes (and they are in warm environments), and by stimulation with bacteria that are not in the VBNC state, by cell-cell communication (*quorum sensing* signaling) [23-26]. Also, it has been detected that cholera outbreaks caused by *Vibrio cholerae* 01 are related to human contact with fresh or saltwater, a medium in which the bacteria can enter or leave the VBNC state depending on the environmental temperature, determined by the seasons of the year [21,22]. The aforementioned are examples of the behavior of VBNC microorganisms in the environment and the importance of their presence for public health.

Conclusion

Bacteria in the VBNC state represent a risk to human health, even in the field of public health. They are often associated with diseases transmitted by water or food. The entry into this state prevents its detection by conventional techniques, based on growth and plate counting, which leads to the underestimation of pathogens since these are the methods that mark the official standards of different countries. Because of this, it is relevant to include complimentary techniques which evaluate the viability of bacteria and not only their cultivability within the regulations that safeguard microbiological safety in different matrixes (beverages, food, biosolids, etc.).

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