A Microbiological Paradox: Viable but Nonculturable Bacteria with Special Reference to Vibrio cholerae

ANWARUL HUQ1 and RITA R. COLWELL1,2*

1Department of Microbiology, University of Maryland at College Park, College Park, Maryland 20742 USA and 2University of Maryland Biotechnology Institute, 4321 Hartwick Road Suite 500, College Park, Maryland 20740 USA

(MS# 95-172: Received 13 July 1995/Accepted 17 October 1995)

ABSTRACT

The observation that directly-detectable bacterial cells are unable to grow on bacteriological culture media under certain conditions raises questions regarding the viability of these cells. Various terminologies have been used to describe substrate-responsive and metabolically-active bacterial cells that cannot be cultured. The currently-accepted term is “viable but nonculturable.” During the past 15 years, the viable but nonculturable phenomenon has been actively investigated. Bacterial pathogens in the viable but nonculturable state can maintain virulence and produce disease. These organisms may escape detection if bacteriological culture methods are solely used. Thus, methods for direct detection of specific pathogens in food, water and environmental samples are preferable. Viable but nonculturable Vibrio cholerae have been extensively studied, and several sensitive and reliable direct-detection kits have been developed. Viable but nonculturable forms of bacteria are now recognized as a common phenomenon, observable in many bacterial species, which suggests that standard bacteriological laboratory protocols for assessing microbiological safety of food and drinking water are less reliable than direct detection methods.

Key words: Viable but nonculturable, Vibrio cholerae

The viable but nonculturable state can be interpreted as one of dormancy, a mechanism for survival and persistence of bacterial cells in the natural or host environment. In this state, bacterial cells maintain viability and metabolic activity, but the cells do not grow and multiply on bacteriological culture plates. A number of factors, such as temperature, nutrient concentration, salinity, osmotic pressure and pH, may be involved in the induction of this state. When bacteria are introduced to a new environment, they are confronted with many changes, but the change in temperature is the most likely to affect the bacteria directly (10). For instance, experimental evidence suggests that increased temperature of Campylobacter jejuni cultivation results in decreased recoverability on culture plates (55). The ability of bacterial cells to respond to shifts in environmental parameters involves a variety of phenotypic and genotypic mechanisms, most likely deriving from evolutionary processes (10, 12). To remain viable, bacteria must utilize both constitutive and inducible enzyme synthesis, accommodate to growth-limiting nutrients and adjust or reroute metabolic pathways to escape metabolic or structural disruption caused by specific nutrient limitations. The adaptability of bacteria in this regard is extraordinary and has been documented by many investigators over the past several decades. In one study, the uptake of 3H-labeled thymidine and 14C-labeled glucose with acetate as substrate decreased as cells of V. cholerae and E. coli entered the VBNC state and dramatically increased upon upshift of temperature from 4°C to 30°C (10). In addition, the ability of the microbial cells to coordinate rates of synthesis to maintain cellular structure and carry out metabolic functions provides bacterial cells with a significant advantage in responding to the surrounding environment (10).

Until recently, the viable but nonculturable phenomenon was described for environmental aquatic pathogens (33) in early studies of Escherichia coli and Vibrio cholerae, both of which are capable of surviving in the viable but nonculturable state (68). During the past decade, clinical and environmental pathogens representing more than 13 genera including the families Enterobacteriaceae, Vibrionaceae and Aeromonadaceae have been reported to occur in the viable but nonculturable state (33). Although it is believed that growth-limiting factors have a major influence on the survival of microorganisms in the viable but nonculturable state, the effect of temperature appears to be extremely important (10, 45, 58). Recently, morphological changes have been demonstrated, in that the cells become coccoid and smaller in size with the central region compressed and surrounded by denser cytoplasm, because temperature change has induced the viable but nonculturable state (12, 39). Organisms not capable of growing on routine bacteriological culture media are considered to be nonculturable, whereas demonstration of viability by direct methods (i.e., measuring the uptake of substrates) will allow determination of viability (4, 10, 56).
BACKGROUND ON VIABLE BUT NONCULTURABLE PHENOMENON

In the literature, various terms have been used to describe the state of bacteria in which the organisms are alive or viable but fail to demonstrate physical evidence of this by forming colonies on culture plates, a method most commonly used to determine the presence or absence of bacteria. The subject of anabiosis, "return to life" or, more appropriately for bacteria, "latent life," intrigued microbiologists. The phenomenon can be traced to the early 18th century; Leeuwenhoek in 1702 recorded evidence for it in his letter "On certain Animalcules found in the sediment in gutters of the roofs of houses" (41). The animalcules referred to by Leeuwenhoek in his letter are now known to have been rotifers. Subsequently, Spallanzani published observations that some microscopic "animals" could be treated with temperature changes, vacuum, electricity, or chemical agents and subsequently revived (60, 61).

The term "anabiosis" was introduced by Wilhelm Preyer (51, 52) for the phenomenon now known as "resuscitation." Valentine and Bradfield (65) described bacterial viability in terms of multiplying and forming colonies, but the designation "live" was suggested also for respiring cells unable to divide under the same conditions. A few years later, Keilin (36) introduced the term "cryptobiosis," that is, latent life, for an organism with visible signs of life but slight metabolic activity. Kurath and Morita (40) also recognized the "live" status of respiring cells, as previously described by Valentine and Bradfield (65). Postgate and Hunter (49), however, suggested such cells were dead if they did not divide, acknowledging that non-dividing bacteria may, in some sense, be alive because they retained their osmotic barrier. Postgate (50) further described the transient state between viability and death when cells exposed to starvation were incapable of multiplication but maintained metabolic function. Thus, according to Postgate, the major criteria for the viability of cells were multiplication and formation of colonies. Years later, Roszak and Colwell (56) coined the term "viable but nonculturable" to describe Salmonella enteritidis detected by direct viable count (38) that was not culturable on bacteriological media. It is evident that viable but nonculturable organisms maintain metabolic activity, indicated by uptake of various metabolic substrates (10).

During the past decade, the term "viable but nonculturable" has been widely used in the literature and applied to bacteria of clinical and environmental origin, the list of which is constantly growing.

IMPLICATIONS

Organisms in the viable but nonculturable state may be found in different stages often indicated by the morphology of the cells. Xu et al. (68) reported changes in the shape and size of E. coli cells entering the viable but nonculturable state. Kondo et al. (39), using the freeze-fixation technique and electron microscopy, demonstrated morphological changes occurring in viable but nonculturable cells of V. cholerae. A sequence of gradual morphological changes in cell structure has been documented in our laboratory using scanning electron microscopy (12). In turn, the morphological changes are correlated with altered nutritional and physico-chemical conditions of the environment. An autoradiographic study of tritium-labeled Helicobacter pylori (58) revealed the aggregation of silver grains associated with the uptake of radiolabeled substrate.

Bacterial cells in the viable but nonculturable state can retain virulence. The first reports of this potential in E. coli was demonstrated by the accumulation of fluid in the ileal loop of rabbits after inoculation with viable but nonculturable cells (14). These findings were corroborated by the observation that virulence plasmids are maintained in E. coli when the cells are in the viable but nonculturable state (9, 21). Other studies showed similar results: clinical symptoms of cholera were manifested in human volunteers after ingestion of viable but nonculturable cells of V. cholerae O1 (15, 19). Viable but nonculturable cells of Vibrio vulnificus have been shown to cause death in mice (46). Virulence of viable but nonculturable cells of Campylobacter jejuni has been demonstrated in rats (57). However, some reports suggest that virulence is lost upon entry of certain cells into the viable but nonculturable state (44). From the investigations reported to date, it appears that most pathogens maintain virulence, suggesting that the careful analysis of food and water samples beyond bacteriological culture alone is needed to assess public health safety.

The waterborne pathogen Vibrio cholerae causes epidemics of cholera in many countries of the world. Serotype O1 of V. cholerae has been identified as the epidemic agent, while the O139 serotype was recognized only recently as having the potential to cause epidemics as well (2). Both serotypes have been shown to enter into the viable but nonculturable state (67, 68). A 3-year study conducted in Bangladesh showed 63% of the plankton samples collected every 2 weeks from ponds and rivers were positive for V. cholerae O1 using the direct fluorescent antibody detection method, yet only 1% were culture-positive (30).1 These natural waters are frequently used for bathing, cooking and drinking. Recently, coexistence of both V. cholerae O1 and O139 was reported in plankton samples in Bangladesh using the direct detection method because the culture method was not always successful (32). A field study was conducted in Bangladesh in which stool samples were collected from patients who were symptomatically confirmed as suffering from cholera. Even these samples did not always yield a positive culture. Furthermore, 8% of the stools which were negative by culture turned out to be positive by Cholera DFA, CholeraScreen, a coagglutination test and a fluorescent antibody test, all of which are direct detection tests now commercially available (23). Some of these culture-negative, DFA-positive stools were further tested by PCR and found to be positive (24).

1 Although only a small percentage of the total number of organisms in a given environmental sample will grow on plates by the culture method, this approach is not likely to be replaced by other methods in the immediate future. However, a sample of clinical origin may yield a higher percentage of recovery than would be expected for environmental samples.
A cholera outbreak, resulting in one death, occurred in California in 1993 among airline passengers arriving from Argentina (1). Stool samples from these passengers were subjected to culture, with less than 100% success. However, vibriocidal antibody titer against *V. cholerae* was significantly high in the blood collected from those patients whose stool samples were culture-negative but positive by Cholera-Screen (1).

A study conducted by Colwell and her colleagues demonstrated that infections resulting from diving in contaminated water do not always manifest clinical symptoms. In this study, infection was determined by detection of raised antibody titers against *Pseudomonas aeruginosa* in post-dive blood samples collected 30 days after a dive and compared with pre-dive blood samples (42) taken when the organism was isolated from the diving site. Elevated antibody titer was detected against *V. cholerae O1* in post-dive blood samples collected from American and Russian divers after diving in waters belonging to their respective countries (31). Culture of *V. cholerae* was not always successful, but the organisms were detected using the direct fluorescent antibody (DFA) method, employing monoclonal antibody. During a field trip to Russia, some of the water and plankton samples collected from the Black Sea were positive for *V. cholerae O1* by culture, and most of the samples revealed the presence of non-culturable cells of *V. cholerae O1* as determined by fluorescent antibody method. Four weeks after this work was completed, an outbreak of cholera occurred in a coastal area where swimming and bathing were permitted. The report appeared in a local Ukrainian newspaper (31).

**OCCURRENCE AND DISTRIBUTION**

Numerous reports of *V. cholerae* being autochthonous, or naturally-occurring, to the aquatic environment (13, 35) are summarized by Colwell and Spira (17) and Blake (6).

Epidemiological data demonstrate that cholera originates in coastal areas and gradually moves inland (3, 63, 64). An hypothetical model of the occurrence and transmission of cholera was proposed by Huq et al. (29) and later modified by Colwell and Huq (18). During the inter-epidemic period, cells of *V. cholerae* convert to the nonculturable state; the number of culturable cells decreases, but it is a drop in the total number of cells that lowers the risk of cholera (30). Quantitative estimation of *V. cholerae* is being investigated in cholera endemic areas during non-epidemic periods. Other enteric diseases such as shigellosis (caused by *Shigella dysenteriae* type 1), *Salmonella* infections, and *E. coli* diarrhea, the most virulent of the latter being O157:H7, are generally transmitted via food. Seafood, however, if contaminated with *V. cholerae* or *V. parahaemolyticus*, can directly cause diarrheal disease. Most cases of cholera in the United States and many cases of diarrhea have been traced to consumption of raw or insufficiently-cooked seafood (5, 43, 47). Vibrios are present in the environment, and environmental water can serve as a source of *V. cholerae*. Consumption of seafood or drinking water containing zooplankton, to which the cholera vibrio is commensal, can transmit the cholera vibrio (33) and potentially lead to a major outbreak or epidemic.

Very little information is available on the presence of viable but nonculturable organisms in food. However, the observations and related published data on waterborne disease leads to the conclusion that pathogens which exist in the viable but nonculturable state may escape detection if bacteriological culture methods are solely employed. In such situations, inaccurate and misleading reports may be generated, preventing public health officials from taking appropriate and necessary action. Thus, it is important to be able to detect both culturable and viable but nonculturable pathogens in food and water.

**DETECTION**

Not only is bacteriological culture the oldest method for detection and identification of bacteria, it is also the most widely-used method worldwide.

Different staining methods, such as acridine orange staining was introduced by Strugger in 1949 (62) to differentiate live cells from dead cells. However, the method is used mainly to enumerate total bacteria in environmental samples, as often the color distinction between live and dead cells is not very clear (7, 20). In light of current knowledge of viable but nonculturable bacteria, there are several sensitive and specific methods of direct detection that have become available during the past few years. A fluorochrome-based staining method employing 4′6-diamidino-2-phenylindole (DAPI) was introduced by Porter and Fieg (48). This staining procedure has become more popular during recent years, and it is the method preferred by environmental microbiologists since it estimates the proportion of total bacterial cells within a specific serotype or taxon when fluorescent DNA probes or fluorochrome-labeled antibodies are combined with a general fluorochrome, e.g. DAPI (37).

Several methods have been developed during the past 20 years for detection of *V. cholerae*. The direct viable count (DVC) developed by Kogure et al. (38) is perhaps the most convincing and widely-used to detect viable cells, irrespective of culturability. In this procedure, viable cells are easily recognized because of enlarged, elongated morphology. This test has been further optimized for detection of *V. cholerae* by combining it with fluorescent antibody (FA) staining. Such a procedure initially used polyclonal antibody and, subsequently, monoclonal antibody developed in our laboratory (8, 68). Using this monoclonal antibody in the FA method, Huq et al. (30) successfully demonstrated the presence of *V. cholerae O1* cells in the natural aquatic environment year-round in Bangladesh, where cholera is endemic. The FA method combined with the DVC method (8) was further developed to permit direct fluorescent antibody-direct viable counting (DFA-DVC), which is now used as a more convenient method for detection of viable but nonculturable cells of *V. cholerae* (11). The DFA-DVC method can be used to detect very small numbers of organisms in food and water samples by concentrating bacteria on filters (27).

The use of soluble p-indonitrotetrazolium (INT) to
demonstrate viable bacterial cells was introduced by Zimmer-
man et al. (69), combined with the Kogure et al. (38) DVC
method and described as the INT-DVC method by Hasan et
al. (22). This method enables detection of viable but noncultur-
able bacteria in water samples, including Aeromo-
nas hydrophila (22) and Shigella dysenteriae (53).
Methods for rapid detection of bacterial cells, especially
viable but nonculturable cells have become very popular
because of the potential public health significance of noncul-
ratable bacteria in food and water. In addition, a recent
cholera outbreak (64) in South America caused by V. choler-
ae O1 and the newly recognized epidemic strain of V.
cholerae O139 (2) has stimulated development of new tests
for the O139 serovar of V. cholerae. Among such tests,
CholeraScreen® (based on coagglutination for detection of
V. cholerae O1 when more than 10⁶ cells per ml are present
on the slide, even in nonculturable state) has proven to be
effective with high specificity and usefulness in both the
laboratory and field (16) for V. cholerae O139 also. Employ-
ing the identical technology of CholeraScreen®, Bengal-
Cholera® has been developed to detect V. cholerae O139
synonym Bengal (24).
A calorimetric immunoassay, SMART® (sensitive mem-
brane antigen detection test), was designed to detect cells in
both the culturable and nonculturable state. It is a colloidal
gold-based, colorimetric immunoassay employing SMART®
technology that binds antigens in a monoclonal and polyclo-
nal antibody sandwich (23). This test, which comes in kit
form and does not require any specialized equipment or cold
storage, demonstrates long shelf-life and lends itself to use
in the field. Two other tests—CholeraScreen (based on
coagglutination, with a monoclonal antibody) and Cholera
DFA (also using a monoclonal antibody tagged with fluores-
coein stain)—are available commercially in kit form from
New Horizon Diagnostics Corporation, Columbia, Mary-
land. However, the latter requires an epifluorescent micro-
scope to complete the test. Based on laboratory testing using
heterologous species and several field evaluations, the DFA
test was found to be 100% specific and 100% sensitive when
compared with culture results (26). Similar results were
obtained for CholeraScreen® and CholeraSMART® (16,
23). Each of these tests has proved useful for both clinical
diagnosis and environmental monitoring of V. cholerae (16,
23, 27). Using the SMART® technology, a test kit from
New Horizon Diagnostics (Columbia, Maryland) has also
been developed to detect E. coli O157:H7 (27a). This test is
currently being optimized for food, water and clinical
samples.
To detect the presence of V. cholerae O1, other methods
such as the cholera-toxin-gene-detecting PCR (25, 59), the
oligonucleotide probe (28), and DNA fingerprinting (54) are
on the horizon with significant potential for wide-scale
application.

CONCLUSION

It is evident from the numerous reports and expanding
literature on the subject that bacterial cells naturally exist in
the viable but nonculturable state, maintaining both viru-

sequence and pathogenic potential, as demonstrated in a variety
of bacteria including V. cholerae. The bacterial cells in the
viable but nonculturable condition can exist in natural
aquatic environments in much larger numbers than hereto-
fore suspected by standard bacteriological plate-culture
methodology (34). Similarly, the presence of various patho-
gens such as Aeromonas, Campylobacter, Shigella, Staphy-
lococcus and Vibrio spp. is usually underestimated by culture
methods (66). Based on current knowledge of the viable but
nonculturable phenomenon, an accurate estimation of bacte-
rial contamination in food is necessary and critical for public
health safety. Appropriate monitoring of environmental and
food sources will allow public health officials to take early
action and implement measures to protect the public. The
reality of viable but nonculturable forms of bacteria, now
recognized as a common phenomenon for many species of
bacteria, demands that bacteriological standards for food
and drinking water be revised to accommodate the newer,
more sensitive and accurate direct detection methods now
available.

ACKNOWLEDGMENT

This work was supported in part by the Thrasher Research Fund,

REFERENCES

799.
Ansaruzzaman, S. M. Faraque and R. Bradley Sack. 1993. Large
outbreak of clinical cholera due to V. cholerae non O1 in Bangladesh.
Lancet. 341:704.
Research, Bangladesh, pp. 36.
strategy of E. coli and Enterococcus faecalis illuminated fresh marine
5. Blake, P. A., D. T. Allegra, J. D. Snyder, T. J. Barrett, L. McFarland,
Feldman. 1980. Cholera—a possible endemic focus in the United
1232.
method for enumeration of viable environmental V. cholerae O1. J.
and pUC8 in non-culturable Escherichia coli in the marine environ-
1995. Physiology and molecular genetics of viable but non-culturable
microorganisms, p. 105–122. In M. Levin, C. Grim and J. S. Angle
(ed.), Biotechnology and Risk Assessment. University of Maryland
Biotechnology Institute, College Park, MD.
R. R. Colwell. 1995. DFA-DVC: A simplified immunofluorescence
technique for detection of viable cells of V. cholerae O1 and O139. J.
Microbiological Meth. In press.
12. Chowdhury, M. A. R., R. Montilla, E. J. Quintero, A. Huq, T. Maugel,
B. Xu, J. A. K. Hasan and R. R. Colwell. 1995. Cellular changes and
VIABLE BUT NONCULTURABLE BACTERIA


