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Journal of Microbiological Methods 53 (2003) 175–183

Journal of Microbiological Methods

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Review article

Molecular methods for the assessment of bacterial viability

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Abstract

A significant number of pathogenic microorganisms can be found in environmental reservoirs (air, water, soil). It is important to assess the viability status of these organisms to determine whether they pose a threat to public health. Classical methods for determining viability are time consuming. Hence, molecular methods have been developed to address this problem. Molecular methods offer speed, sensitivity and specificity. Both DNA and RNA have been analysed using molecular amplification methods such as polymerase chain reaction (PCR), reverse transcriptase PCR (RT-PCR) and nucleic acid sequence-based amplification (NASBA). However, due to the variable persistence of nucleic acids in cells post-death, the correlation between presence of DNA and RNA and viability is not clear-cut. Similarly, the choice of target and sensitivity of the method can significantly affect the validity of the viability assay. This review assesses the molecular methods currently available and evaluates their ability to assess cell viability with emphasis on environmental pathogens.
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Keywords: Bacterial viability; Persistence; PCR; RT-PCR; NASBA; Nucleic acid amplification

1. Introduction

Classical methods for the determination of bacterial viability rely on the ability of cells to actively grow and form visible colonies on solid media. Under some circumstances, the number of viable organisms may be severely underrepresented by such methods as sublethally damaged organisms (Blackburn and McCarthy, 2000), fastidious uncultivable bacteria (Ward et al., 1990) and viable cells that have lost the ability to form colonies under the test conditions will not be detected. Consequently, alternative methods for determining viability have been developed, based variously on demonstration of cellular integrity or activity. Alternatives to colony counting include the use of flow cytometry and fluorescent staining techniques (Caron et al., 1998; Diaper and Edwards, 1994; Turner et al., 2000), the exploitation of physiological responsiveness (Kogure et al., 1979) or metabolic activity and nucleic acid-based analyses (del Mar Lleó et al., 2000; McCarty and Atlas, 1993; Sheridan et al., 1998). Fig. 1 shows a schematic diagram illustrating the range of techniques used in assessment of bacterial viability status. For the purposes of this review, cells maintaining membrane integrity and retaining some metabolic activity or responsiveness are considered viable.

The analysis of environmental bacteria is performed for a variety of applications, ranging from the assessment of microbial diversity to monitoring pathogen presence for public health protection. A number of

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0167-7012/03/$ - see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0167-7012(03)00025-3

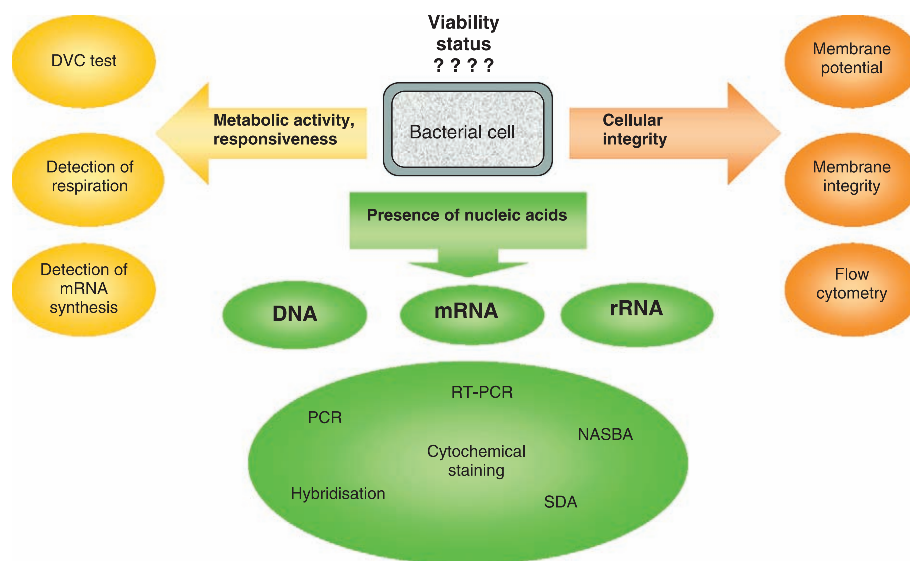

Fig. 1. Schematic diagram illustrating the range of approaches used in the assessment of bacterial viability.

significant bacterial pathogens may be present in environmental reservoirs, including Verotoxigenic *Escherichia coli* (VTEC), *Vibrio vulnificus*, *Salmonella* spp., *Campylobacter* spp. and a variety of mycobacteria. Analyses for the detection of these and other pathogens in water supplies, in soil and as airborne contaminants have been developed. To determine if the presence of these pathogens poses an actual threat to public health, their viability needs to be determined, although the consistent detection of nucleic acid sequences from pathogenic bacteria in an environment can be taken as indicative of a hazardous contamination (Moore et al., 2001). The development of rapid, sensitive and specific methods for the detection and identification of environmental bacteria with concomitant determination of their viability status would significantly improve current surveillance regimes.

2. Molecular targets for assessment of viability

A range of molecular targets have been utilised in microbiological assays. Experiments utilising the polymerase chain reaction (PCR) have been used to detect DNA sequences and identify and enumerate bacterial species. More recently, the use of DNA microarrays (Ye et al., 2001) and PNA probes (Stender et al., 2002) has been employed to increase the speed and specificity of detection of bacterial species present in a sample. However, these assays do not give any indication as to viability of the organisms. The presence of intact DNA sequences was initially used as an indicator of cell viability with the assumption that the DNA would be degraded in a dead cell more rapidly than other cellular components (Jamil et al., 1993). Similarly, the detection of longer intact DNA sequences correlates more closely with viability than shorter sequences (McCarty and Atlas, 1993). However, the precise correlation of cell viability with detection of DNA was shown to be poor, with DNA persisting in actively killed cells for significant periods of time (Masters et al., 1994). DNA was also demonstrated to persist in a PCR-detectable form in culture-negative environmental (Deere et al., 1996) and clinical samples (Hellyer et al., 1999). Attention turned to the use of mRNA as a marker of viability, as mRNA is a highly labile molecule with a very short half-life (seconds) and, therefore, should provide a more closely correlated indication of viability status than DNA-based methods.

Ribosomal RNA (rRNA) has also been investigated as an indicator of viability (McKillip et al., 1999; Villarino et al., 2000) and has been found to be positively correlated with viability under some bacterial-killing regimes (McKillip et al., 1998). In addi-

tion, detection of 16S rRNA from *Chlamydia pneumoniae* was demonstrated to provide a more suitable indication of active infection than immunocytochemical detection of specific antigens (Meijer et al., 2000). However, the longer half-life of rRNA species and their variable retention following a variety of bacterial stress treatments (Tolker-Nielsen et al., 1997) make rRNA, under many conditions, a less accurate indicator of viability than mRNA targets.

3. Impact of analytical method

The majority of current molecular analyses for viability utilise target amplification to maximise analytical sensitivity. Most DNA detection is undertaken using PCR (Hellyer et al., 1999; McKillip et al., 1999), although hybridisation-based detection methods have also been employed (Meijer et al., 2000). The most commonly used amplification techniques for detecting mRNA are reverse transcriptase PCR (RT-PCR) and nucleic acid sequence-based amplification (NASBA) (Chan and Fox, 1999; Simpkins et al., 2000). Both these methods have been applied to the determination of bacterial viability, with variable success. More recently, reverse transcriptase-strand displacement amplification (RT-SDA) has also been used as an indicator of bacterial viability (Hellyer, 2001).

RT-PCR is a two-stage process, in which a target messenger RNA sequence is first transcribed into a complementary DNA (cDNA) sequence, either using random hexanucleotide primers or sequence-specific primers. The cDNA sequence may then be used to generate a second-strand cDNA, or serve directly as a template for a PCR, resulting in an exponential amplification and subsequent detection of the original complementary target. NASBA is a transcription-based amplification reaction that utilises three enzymes to mimic retroviral replication (Chan and Fox, 1999). Specifically, avian myeloblastosis virus reverse transcriptase (AMV-RT), ribonuclease H (RNase H) and T7 RNA polymerase act together to amplify an RNA target in an isothermal reaction, which proceeds exponentially to amplify multiple antisense copies of the RNA target. RT-PCR has historically been the amplification method of choice when analysing RNA, mainly as PCR has become established as a key technique underpinning many DNA-based measurements. In addition, the reverse transcriptase enzymes are widely available in highly purified forms. However, the cost of licensing PCR has led to the development of alternative amplification techniques, including NASBA and strand displacement amplification (SDA). NASBA offers several advantages over other mRNA amplification methods. NASBA is an isothermal reaction performed at 41 °C, which obviates the need for a thermal cycler and may facilitate the production of point-of-test devices. A single-stranded antisense RNA product is produced during NASBA, which can be directly hybridised by a probe sequence to accelerate post-amplification interrogation of the product. Importantly, for preanalysis sample preparation, RNA targets cannot be specifically analysed by RT-PCR in a background of contaminating DNA. To overcome this, either intron-flanking primers must be designed or DNA contamination removed enzymically by DNases. In contrast, background DNA does not interfere with the NASBA reaction, as single-stranded RNA sequences are specifically targeted (Simpkins et al., 2000). Thus, for environmental analyses, use of NASBA removes the necessity for specific RNA extraction procedures; although, obviously, maintaining the integrity of RNA species is necessary for successful analysis using both RNA amplification methods.

The sensitivity of the detection method utilised will determine the level at which the persisting nucleic acids can be detected, and thus, may significantly affect the perceived viability status of a cell. In our hands, we have found NASBA amplification to be more sensitive than RT-PCR of equivalent targets, and in a recent study of the efficacy of PCR, RT-PCR and NASBA in assessing bacterial viability found that while RT-PCR apparently correlated more closely with culturally determined viability than NASBA, this was due to the lesser sensitivity of the RT-PCR analysis (Birch et al., 2001). In several studies, it has been shown that both DNA and mRNA species may persist in a detectable form for many hours after cell death (Sheridan et al., 1998, 1999; Birch et al., 2001), confirming the potential for poor correlation between mRNA detection and cell viability. A further implication is that the increasing sensitivity of nucleic acid-detection methods may widen the observed discrepancy between actual viability and nucleic acid-assessed viability status.

4. Detection and significance of viable but nonculturable bacterial populations

Methods for the determination of viability depend on examination of a variety of cell characteristics. However, the definition of what constitutes a viable cell remains contentious, with the significance and precise physiological status of nonculturable organisms that retain some sign of viability, so-called VBNC forms, still undetermined (Barer and Harwood, 1999; Kell et al., 1998). A number of bacterial species have been observed to undergo metabolic change leading to the production of cells that can no longer actively form colonies on solid media, but that retain other indicators of cell viability, such as active membrane potential, maintenance of cellular integrity and the capacity for metabolic activity (Besnard et al., 2000; del Mar Lleó et al., 2000; Grey and Steck, 2001b; Nilsson et al., 1991; Turner et al., 2000). A number of events have been shown to trigger this observed loss of culturability, such as temperature shift-down, nutrient deprivation and exposure to toxic agents, and thus, diverse physiological phenomenon may be attributed to the VBNC state. Indeed, it has been demonstrated that populations of unculturable *V. vulnificus* could instead be sublethally injured hydrogen peroxide-sensitive culturable cells (Bogosian et al., 2000).

It remains to be determined whether cells in the nonculturable state retain pathogenicity (Barer et al., 2000; Grimes et al., 1986). Studies have shown that VBNC *Salmonella* cells can neither infect nor colonise mice, even at doses significantly higher than the normal LD_{50} (Barer et al., 2000). Other researchers have demonstrated that the VBNC form of *Legionella pneumophila* failed to cause disease in an animal model; although, resuscitated cells regained their virulence and could then cause infection (Steinert et al., 1997). A more recent study has shown the potential of a plant pathogen, *Ralstonia solanacearun*, to be resuscitated by the proximity of an appropriate host and to subsequently effect infection (Grey and Steck, 2001a). Similarly, studies using injection into embryonated eggs to recover VBNC *Campylobacter jejuni* have indicated that resuscitated cells regain the ability to attach to HeLa cells, a measure of pathogenicity (Cappelier et al., 1999). In this study, dilute populations of cells were utilised for the recovery experiments, demonstrating clearly that resuscitation of nonculturable cells rather than regrowth of a small population of viable cells had occurred. It has also been demonstrated that genes encoding virulence determinants are retained for long periods in cells that have lost culturability (Chaiyanan et al., 2001). Thus, although the ability of VBNC forms to cause disease has not been absolutely demonstrated, nonculturable pathogens may still pose a hazard to public health.

Molecular methods cannot easily differentiate between culturable and VBNC forms, as the heterogeneous nucleic acid content of cells within VBNC populations, the gradual nature of DNA and RNA degradation, and variable persistence of nucleic acids dependent on environmental conditions does not permit simple analysis. Studies of stationary phase cells have demonstrated stabilisation of the mRNA pool concomitant with shift-down of metabolic activity (Thorne and Williams, 1997; Smeulders et al., 1999). Direct analysis of nucleic acids in VBNC cells has shown their persistence to be variable. Several studies have shown evidence of long-term persistence of DNA and RNA (Lázaro et al., 1999; del Mar Lleó et al., 2000), while other research has demonstrated the amount of nucleic acid to gradually decrease (Weichart et al., 1997), with the DNA content detectable by PCR decreased by several orders of magnitude (Brauns et al., 1991). In addition, analysis of the DNA content of planktonic bacteria has allowed classification of cells as either high or low DNA forms, with good correlation between high DNA content and the ability to respond to environmental changes (Gasol et al., 1999). Definitive nucleic acid and physiological characterisation of VBNC cells need to be established to allow accurate molecular identification. Further, to permit practical interpretation of molecular analyses of environmental pathogens, the infectivity of such nonculturable populations that retain some metabolic activity or responsiveness must be determined.

5. Molecular methods for pathogen detection

The main applications where determination of viability is central to the analysis are associated with detection of pathogens, either in environmental samples or clinical situations. Analysis of environmental

samples includes the investigation of air and water quality, and assays have been developed to detect many significant human pathogens. A recent review considering the use of integrated biodetection systems for investigation of uncultivated environmental micro-organisms has stressed the need for processes tailored to the unique aspects of environmental samples (Chandler, 2002).

To comply with regulatory requirements, water service providers must ensure that drinking water supplies are adequately monitored. Methods for analysing water quality are designed to detect the presence of faecal coliforms as indicators of water quality (Rompre et al., 2002). Routine analysis methods in the water industry are mainly based on immunofluorescence and detection of enzyme activity (Reynolds and Fricker, 1999), some of which has the ability to distinguish live and dead organisms. Molecular analyses are mainly utilised as research tools rather than for routine surveillance, although array-based and microchip-based methods with high throughput potential are currently under development. A novel method utilising NASBA amplification of a mRNA produced in response to heat shock by the protozoan pathogen *Cryptosporidium parvum* combined with liposome-mediated signal amplification on a microfluidic chip has also recently been described (Esch et al., 2001). The method relies on detecting the ability of viable *C. parvum* oocysts to respond to heat stress by synthesis of heat-shock protein mRNA, utilising NASBA amplification of a specific mRNA target. The amplified mRNA is hybridised between capture probes immobilised in a microfluidic channel, and reporter probes labelled with liposomes containing up to 10^5 fluorescent molecules in each liposome. This method provides a significant increase in sensitivity compared to single fluorophore labels, and the general approach may be extended to the detection of bacterial heat-shock responses.

A recent study has utilised RT-PCR directed to a heat-shock transcript, to enable detection of viable *Pneumocystis carinii* (Maher et al., 2001). The specificity of the assay for viable organisms was demonstrated by comparison of samples from infected individuals before and after autoclave inactivation of the bacteria however; and consequently may not be a good indicator of the performance of the method for environmental viability studies. mRNA species may persist in a detectable form far longer in cells that have undergone natural death and decay processes, in comparison to autoclaved samples.

In clinical analyses, detection of the load of viable pathogenic species is used to assess the efficacy of antimicrobial therapy and persistence of infection during treatment. Molecular assays have been developed to detect the presence of mRNA species from *Mycobacterium tuberculosis* (Hellyer et al., 1999; Jou et al., 1997), and have demonstrated that using an abundant transcript there was good correlation between detectable mRNA species and bacterial viability. In some clinical settings, the effective use of PCR to monitor the persistence of viable organisms has been demonstrated, for example, in the analysis of *Borrelia burgdorferi* in synovial fluid samples (Priem et al., 1998; van der Heijden et al., 1999). Thus, both DNA and RNA analyses can be used as effective measures of viability in certain situations.

6. Molecular methods for ecological studies

Molecular methods have been introduced for the assessment of microbial diversity, contributing specificity of bacterial identification and sensitivity to such analyses. Viability of environmental bacteria is mainly determined by direct viable count (DVC) (Kogure et al., 1979) and staining methods (Bernard et al., 2001; Miskin et al., 1998). However, the presence of specific mRNA transcripts in RNA libraries produced from environmental samples has been used as an indicator of active microbial populations (Miskin et al., 1999). Novel array technology has been exploited to allow large-scale multiplex analysis of environmental gene pools (Wu et al., 2001); the advantage of such analyses is the potential to analyse multiple targets simultaneously. Further, array technology has been integrated into multifunctional systems for field-based studies (Bavykin et al., 2001); although, to date no array-based assays that enable discrimination between viable and nonviable bacteria have been developed.

The sensitivity, specificity and rapidity of molecular methods have also led to their use for bioaerosol monitoring in determination of air quality and the detection of airborne pathogens (Alvarez et al., 1994; Buttner et al., 2001). However, most molecular anal-

yses of air quality are directed at bacterial identification rather than determination of viability.

7. Reliability of molecular analysis of bacterial viability

The detection of both DNA and RNA species has been used as indicators of bacterial viability. However, these are indirect methods, and as the persistence of nucleic acids is highly dependent on the environmental conditions encountered by the bacterium after cell death (Romanowski et al., 1992; Sheridan et al., 1999), the correlation with actual cell viability may vary greatly. Similarly, many of the staining techniques used for the assessment of bacterial viability are indirect analyses, monitoring variously membrane potential (Rhodamine 123 and $DiBAC_4(3)$), membrane integrity (SYTO 9/propidium iodide/GFP persistence) (Lowder et al., 2000; Banning et al., 2002), and DNA and RNA persistence (SYTO dyes). More direct indicators of viability are the detection of respiratory activity (CTC), environmental responsiveness (Nwoguh et al., 1995), substrate responsiveness (DVC assay) and culturability. Of the current methods available for monitoring viability, no single physiological indicator is universally appropriate, and utilisation of several indicators offers the potential for increased accuracy of characterisation (Lisle et al., 1999).

For molecular methods, the choice of target can significantly affect the validity of correlation with viability. Using PCR analyses, it has been demonstrated that longer targets are more indicative of viability than shorter amplicons (McCarty and Atlas, 1993). In mRNA analysis, there is the added consideration of choice of target transcript. The use of ubiquitously expressed sequences should ensure that the transcript is present under most assay conditions, and highly expressed targets may provide more sensitive analyses (Sheridan et al., 1999). A recent investigation of a number of target genes as indicators of viable *E. coli* O157:H7 in unculturable, heat-shocked or heat-killed populations demonstrated that choice of target was important in providing a reasonable correlation with cell viability (Yaron and Matthews, 2002). The region of the specific transcript targeted may also have an impact on the performance of the analysis as an indication of viability. A study utilising the 5′ nuclease assay has demonstrated that primers directed to the distal region of the *Listeria monocytogenes hly*A gene provided good correlation with viability, while more centrally located primers overestimated the number of viable cells (Norton and Batt, 1999). In theory, mRNA analysis should provide a good indication of bacterial viability, as mRNA species persist for only short periods of time in actively metabolising bacterial cells with an average half-life of minutes (Arraiano et al., 1988). However, mechanisms of prokaryotic mRNA decay are complex and degradation pathways are only now being elucidated (Regnier and Arraiano, 2000; Steege, 2000). Such variation highlights the need for some knowledge of the persistence and decay characteristics of the potential mRNA target of an assay, to enable design of an accurate functional assay for viability.

8. Future prospects

Many currently used methods for the determination of bacterial viability actually monitor decreasing levels of signal during cellular degradation after cell death. Molecular methods detect decaying nucleic acids, and both DNA and RNA persistence have been shown to correlate well with viability in certain well-characterised situations. Similarly, analysis of membrane integrity is used as a secondary indicator of cell death. All such indirect analyses rely on rapid clearance of dead cells to ensure reliable correlation of detected signal with cell viability, and hence, the accuracy of such methods is highly dependent on both the manner of cell death and environmental conditions. Utilisation of several indirect methods will provide a better correlation with cell viability than reliance on any single analytical technique.

A better indicator of cell viability would be an analysis in which an active response is elicited and detected as a direct measure of viability. Detection of metabolic responsiveness is the basis of the DVC approach to enumeration of viable organisms, and microscopic methods for the detection of responsiveness by gene expression have also been utilised to demonstrate bacterial viability (Barer, 1991; Lewis et al., 1994). The development of methods for the analysis of transcriptional responsiveness should per-

mit more direct molecular analysis of bacterial viability (Esch et al., 2001). Detection of gene induction or transcriptional responsiveness could be achieved by either analysis of representative individual cells (by microscopy or flow cytometry for example), or by quantitative analysis of differences in transcript levels before and after induction. Coupling the quantitative approach with array-based detection systems to allow the simultaneous analysis of multiple targets could be used to provide an effective monitoring system for the identification of viable cells.

Acknowledgements

This work was funded by the Department of Trade and Industry under the Valid Analytical Measurement (VAM) 2000–2003 programme. We thank Dr. Gavin Nixon and Carol Dawson for useful discussions during preparation of this manuscript, and the referees for their helpful suggestions.

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