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Assessment of bacterial viability: a comprehensive review on recent advances and challenges

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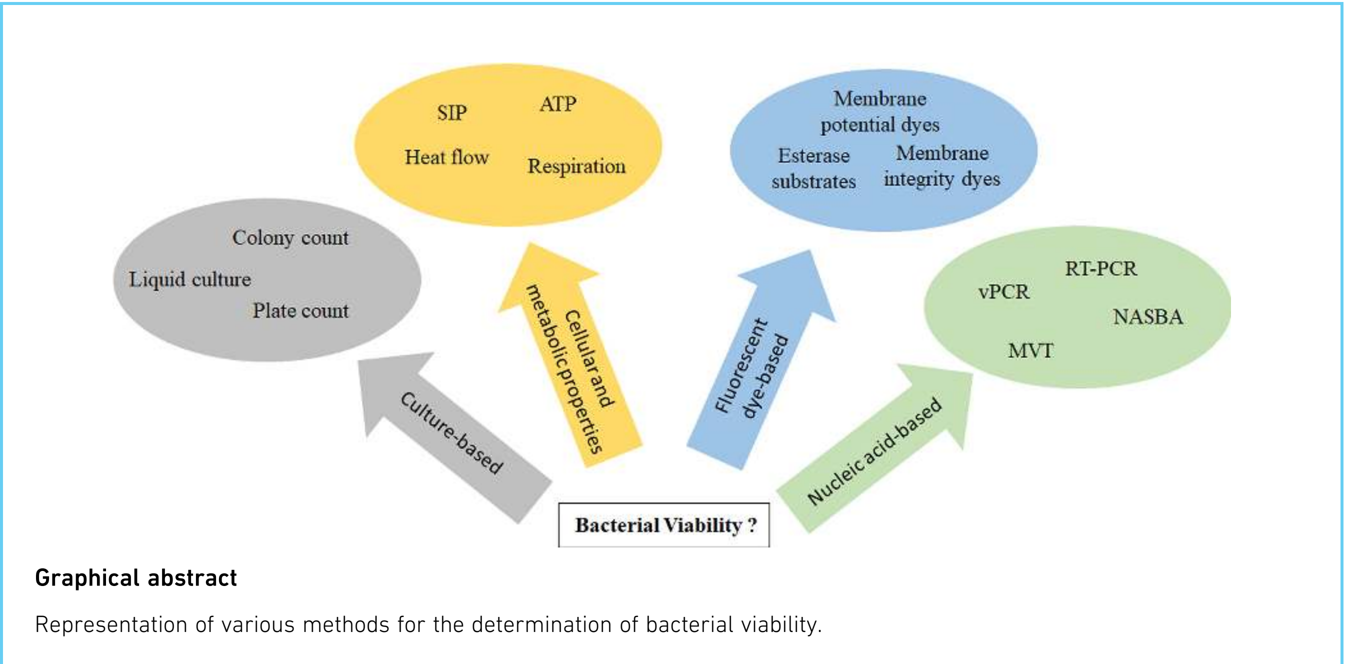

Graphical abstract

Representation of various methods for the determination of bacterial viability.

Abstract

Assessing bacterial contamination in environmental samples is critical in determining threats to public health. The classical methods are time-consuming and only recognize species that grow easily on culture media. Viable but non-culturable (VBNC) bacteria are a possible threat that may resuscitate and cause infections. Recent dye-based screening techniques employ nucleic acid dyes such as ethidium monoazide (EMA) and propidium monoazide (PMA), along with many fluorescent dyes, which are an effective alternative for viability assessment. The measurement of cellular metabolism, heat flow and ATP production has also been widely applied in detection approaches. In addition, RNA-based detection methods, including nucleic acid sequence-based amplification (NASBA), have been applied for bacterial pathogen determination. Stable isotope probing using ^{13}C, ^{15}N and ^{18}O, which are mobilized by microbes, can also be used for effective viability assessment. Future detection tools, such as microarrays, BioNEMS and BioMEMS, which are currently being validated, might offer better microbial viability detection.

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Keywords: Viability; VBNC; vPCR; MVT; Membrane integrity.
Abbreviations: ahpC, alkyl hydroperoxide reductase subunit C; AMV, avian myeloblastosis virus; DMSO, dimethyl sulfoxide; DOC, deoxycholate; DVC, direct viable count; EMA, ethidium monoazide; GST, glutathione S- transferase; IMC, isothermal microcalorimeter; IMS, immunomagnetic separation; LOD, limit of detection; MVT, molecular viability test; NASBA, nucleic acid sequence-based amplification; PI, propidium iodidE; PMA, propidium monoazide; (p)ppGp, guanosine 3′, 5′-bispyrophosphatse; RpoS, RNA polymerase sigma S; SIP, stable isotope probing; VBNC, viable but non-culturable.

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INTRODUCTION

Determining microbial viability is crucial in the field of microbiology and yet the criteria for determining viability remain ambiguous. The viability of microbes is key in determining the safety of food and drinking water and has severe implications in the fields of environmental and medical microbiology [1]. Bacterial pathogens are the most common causes of infectious diseases worldwide. The common bacterial infectious diseases, such as diarrhoea, cholera, dysentery and pneumonia, are a major threat to many socio-economically disadvantaged countries, such as Asia–Pacific (APAC) and African countries, and hence the detection and elimination of their causative agents or pathogens should be the primary focus of research [2].

Growth and division is a widespread and accepted parameter for the detection of bacterial viability. The culturabilty of bacteria is generally ascertained by determining their ability to propagate in liquid nutrient medium or in solid culture medium, with them appearing as visible colonies. Culture-based methods have proven to be efficient and cost-effective over many years. However, assessing the viability of mesophilic heterotropic bacteria by agar plate methods requires overnight incubation and furthermore this technique does not provide any information about bacterial physiology. Intact cell membrane, metabolic activity and reproducibility are the three accepted general parameters for the assessment of the viability of microbes [3]. Culture-based methods only account for one of these parameters, i.e. reproducibility. Absence of growth in culture-based methods does not always mean non-viability, it may also be interpreted as: (i) incorrect culture medium; (ii) stress or damage to cells leading to a dormant state or injured state; (iii) low population density, due to which there has been no observable growth; and (iv) slow-growing cells and hence no visible growth [4].

However, many bacterial species have been found to exist in the viable but non-culturable (VBNC) state. The VBNC state is characterized by the loss of culturability on routine agar media and thus reduces the chance of detection by the conventional plate count technique. This leads to the underestimation of total viable cells in environmental and clinical samples and in turn may prove to be a threat to public health. Pathogens in the VBNC state are known to retain virulence and can be resuscitated to initiate infection under favourable conditions [5]. A number of foodborne outbreaks have been associated with the resuscitation of pathogens that were present in the VBNC state [6, 7]. Many bacterial human pathogens known to cause disease have been proven to exist in the VBNC state, with each species having different induction conditions, resuscitation factors and mechanisms [8]. Hence the detection of pathogens in the VBNC state is crucial for the prevention of outbreaks. A proper detection system is still required to eliminate or reduce this gap in pathogen detection, and this should be more efficient and practicable than the traditional system that has been followed until now.

This article discusses the properties associated with the VBNC state of bacteria; its induction and resuscitation conditions; and the factors responsible for maintaining it. The article further assesses alternative methods to overcome the drawbacks of culture-based methods in determining bacterial viability and discusses the possible application of these methods in different environmental conditions. The advances and challenges associated with different molecular methods, including dye-based as well as cellular property-based methods, are discussed to aid in the selection of the most suitable technique.

EVALUATION OF THE CULTURE-DEPENDENT TECHNIQUE

Culture-based detection of microbial contamination involves a pre-enrichment step wherein the sample is inoculated in a non-selective medium. This helps in the proliferation of pathogens that are present in low numbers, stressed or injured, and improves detection [9]. Further inoculation in several selective media allows the growth of the target organism while suppressing others, and this is followed by enumeration and biochemical screening. Culture-based detection cannot be applied to all bacterial species, as in the case of spirochaete, *Treponema pallidum,* the causative agent of syphilis, or *Mycobacterium leprae,* the pathogen for leprosy, which has remained unculturable to date and is undetectable by culture-based methods *in vitro* and is therefore propagated through rabbits and armadillos for research [10]. Furthermore, many bacterial species may not grow in culture media when (i) growth requirements and incubation conditions are not fulfilled by artificial media; (ii) competition persists for nutrients among many bacterial species in a mixed culture, inhibiting growth; (iii) the presence of bacteriocin from other bacteria in mixed culture leads to inhibition of growth of the target species; and (iv) signalling molecules and beneficial interactions essential for growth of target species are absent [11]. Such bacterial species are termed unculturable and cannot be detected by culture-based methods. Culture-based methods operate through trial and error, and species are ruled out one after the other based on their biochemical and physiological properties until a final answer is reached [12]. Interpreting the results of culture-based methods requires technical skills, especially when there are non-conforming results or there is a new strain with non-conforming properties. Ascertaining the presence of a pathogen in samples takes many days using culture-dependent methods; for example, it takes 7–10 days to obtain a positive detection result for *Campylobacter* species [13].

Viable but non-culturable: a limitation of the culture-dependent technique

Microbes that fail to cultivate on routine media on which they normally grow and yet are still alive are said to be in the VBNC state [14]. The VBNC state was first reported by Xu *et al.* [15]. Many species of bacteria when under stress may enter a dormant state to survive and do not grow on

Table 1. Characteristics of VBNC cells

Serial no.	Traits	Description	Reference
1	Cell morphology	VBNC cells have intact cell membranes and have high membrane potential. Dwarfing of cells or miniaturisation; rod-shaped cells become coccus. Peptidoglycan become more cross-linked, lipoprotein content is enriched and glycan strands reduce in length, which may lead to changes to the shape of the cell	[20, 155–158]
2	Energy usage	Total cellular content of carbohydrate and lipid and polyhydroxybutrate is reduced, which suggest their use as energy sources	[159]
3	Translation	Continued uptake of amino acids and incorporation into proteins	[160]
4	Cellular ATP	ATP levels remain higher than those of normal cells	[161, 162]
5	Respiration	Reduced respiration rate and metabolic rate	[161, 163]
6	Genomic integrity	Chromosomal DNA integrity is retained even after the loss of culturability	[164]
7	Gene expression	Gene expression profiles are different compared to those of culturable cells. A study of *V. cholera* VBNC cells showed upregulation of genes related to regulatory functions, cellular processes, energy metabolism, transport and binding, and downregulation of genes related to protein synthesis and stress response	[23, 165–167]
8	Virulence	Most species have been reported to be avirulent in the VBNC state, but they express virulence when resuscitated under favourable conditions. *Listeria monocytogenes*, *Vibrio harveyi*, *Vibrio vulnificus*, *Vibrio alginolyticus 1* and *Vibrio parahaemolyticus 66* have been reported to express virulence after resuscitation under favourable conditions	[39, 168–170]
9	Stress tolerance	VBNC cells are tolerant to stress, such as antibiotics, heavy metals, high temperature, high salinity, ethanol and acid. This may be because of the lower metabolic rate and increased cross-linking of peptidoglycan. VBNC cells have been shown to be more resistant to sonication [171], high temperature [172], low salinity, low pH, ethanol, chlorine and antibiotics than cells in growing in the exponential phase [173]	[14, 174–176]
10	Resuscitation	VBNC cells resuscitate rapidly in host cells	[169, 170, 177]
11	Adhesion	The adhesion property changes in VBNC cells; some species, such as *C. jejuni*, retain their adhesion property, but species such as *V. cholera* show reduced adhesion properties, and *E. faecalis* is unable to attach and form biofilm	[178–180]

culture media but retain metabolic activity and may return to their virulent state in the presence of nutrition or after the removal of stress. The factors that induce VBNC are starvation [16], oxidative stress [17], oxygen limitation [18], osmotic pressure, chemicals/disinfectants, low pH, temperature fluctuations, pulsed electric field and aerosolization [19]. Bacteria in the VBNC state may be assumed to be non-viable by standard culture methods, but differ vastly from dead bacteria. VBNC bacteria deviate from conventional microbial growth characteristics and hence cannot be detected by traditional plating methods. VBNC cells have an intact cell membrane and maintain the membrane potential, unlike dead cells, whose membranes have disintegrated [20]. There is a constant debate as to whether the VBNC state is just cells adapting to non-favourable conditions or in fact cells in the state preceding death [21].The VBNC cells of different species have been extensively studied and all the results suggest that the characteristics of VBNC cells are vastly different from those of dead cells (Table 1). Further, studies have shown that VBNC cells can be resuscitated under favourable conditions [22]. Traditional culture methods are not favourable for the determination of VBNC cells, hence alternative methods to detect VBNC cells are very important for the detection of contamination in various environmental conditions.

Induction of VBNC cells

VBNC cells that are pathogenic and known to cause human diseases pose a major threat as they can be avirulent and undetected by conventional diagnostic methods and subsequently resuscitate and cause infections. Therefore understanding the mechanisms involved in the induction of the VBNC state in bacteria as well as subsequent resuscitation is vital for the prevention of bacterial infections. Bacteria from diverse species and different environmental conditions have been known to enter the VBNC state under various stressors, which suggests that this state is a widespread mechanism adopted by bacteria to overcome unfavourable conditions [23]. Gene expression during stressful conditions and during the VBNC state can help us to determine which genes influence the induction of the VBNC state. RpoS, a stress regulator, is one such gene which was found to regulate the VBNC state [23].

RpoS is a sigma factor that is responsible for the bacterial survival in the stationary phase as well as in stressful conditions [19]. RpoS controls the expression of a group of specific genes by interaction with RNA polymerase (RNAP) and is known to regulate 10 % of the *Escherichia coli* genome [24]. RpoS levels are low in the growth conditions of bacterial cells, and as the cells enter the stationary phase or stressful conditions there is an increase in RpoS levels, which induces an RpoS response [24]. Many regulatory pathways have been studied that are known to signal the RpoS response, among which (p)ppGpp (guanosine 3′, 5′- bis-pyrophosphate) has been found to be indicative [25]. With an increase in (p)ppGpp there is increase in rpoS expression and a reduction in RpoS degradation, as well as an increase in RpoS activity [23]. RelA, a monofunctional alarmone synthase, and SpoT, a bifunctional synthase as well as

hydrolase, regulate the (p)ppGpp in beta and gamma proteobacteria [26]. Regulation of (p)ppGpp regulates RpoS and accumulation of (p)ppGpp leads to enhanced stress resistance in VBNC cells [23].

OxyR is a transcriptional regulator that is known to regulate oxidative stress-related genes and was first discovered in *Salmonella typhimurium* by Christman *et al.* [27]. *E. coli* in the VBNC state has been shown to have decreased superoxide dismutase (SOD) activity leading to oxidative damage, which suggests that the oxidative stress response is involved in the induction and maintenance of the VBNC state [28]. Antioxidative enzymes such as alkyl hydroperoxide reductase subunit C (ahp C) and glutathione S-transferase (GST) have been shown to influence the induction of the VBNC state in *Vibrio* species [29, 30]. In turn, OxyR regulates ahp C in response to increased ROS levels [31] and GST in response to organic peroxides [32], hence regulating the induction and maintenance of the VBNC state [23].

Resuscitation of VBNC cells

The reversal of the changes leading to the formation of VBNC cells through the removal of stressors is termed resuscitation [33]. The resuscitation of non-culturable bacteria was first reported in *Salmonella enteritidis* after supplementation with heart infusion broth [34]. Studying the resuscitation properties is tedious, as it is difficult to differentiate between cells that have resuscitated from the VBNC state and residual normal cells that are under the limit of detection [35].

Resuscitation has been proven in many human pathogens under different conditions, e.g. *Salmonella enteritidis* after incubation with catalase in M9 minimal media [36] and *Campylobacter jejuni* in embryonated chicken eggs [23]. The conditions for resuscitation are different for each bacteria and the removal of stress induction alone may not lead to resuscitation. VBNC cells of haemolytic *E. coli* were able to resuscitate in the presence of amino acids but VBNC cells of *E. coli* O157:H7 could not be resuscitated under the same conditions, which suggests that resuscitation conditions differ among strains of same species [37]. Temperature increase, sodium pyruvate [5], amino acids [37] and Tween 20 [38] have been reported to mediate the resuscitation of VBNC cells. Resuscitated bacterial cells of *Listeria monocytogenes* [39] and *Salmonella typhi* [33] have been shown to exhibit virulence and cause infections and death in mice.

CULTURE-INDEPENDENT TECHNIQUES

Fluorescent dyes

Cell viability can be detected by using the characteristics of viable cells, such as enzyme activity, which may result in substrate uptake and cleavage, cell energy and integrity of the cell membrane [40]. These properties of viable cells can be detected using a microscope or flow cytometry with the help of fluorescent dyes. Some of the dyes used in viability detection are listed in Table 2.

Membrane integrity using nucleic acid dyes

Membrane integrity is an accepted biomarker for viable cells, as cells with compromised membrane are approaching death or already dead [41]. There are numerous ways to detect the membrane integrity of cells and in turn the viability of cells. The structure of the cell membrane and its composition vary widely among bacteria, hence analysis of viability using membrane integrity as a biomarker is challenging in mixed cultures or in environmental samples [40]. Polar stains do not permeate into viable cells with intact membranes but penetrate into dead cells with compromised membranes [42]. There are two types of dye that can be used to determine membrane integrity. (i) Dyes that can permeate into intact and compromised cells, such SYTO 9, Hoechst 33 342 and acridine orange. (ii) Dyes that can only permeate compromised cells, such as propidium iodide, ethidium homodimer, SYTOX Blue, SYTOX Green, YOYO-1, TOTO-1 and TOPRO3 (77). Cell-permeant dyes bind to the DNA of live cells, while cell-impermeant dyes bind to the DNA of dead cells. Dual-staining kits such as the LIVE/DEAD BacLight Bacterial Viability kit use two fluorophores, SYTO9 and propidium iodide (PI). The distinction between live and dead is based on membrane integrity, as PI, a red fluorescent nucleic acid stain, only enters cells with compromised membranes and SYOT 9 enters all cells and binds to DNA. SYTO 9 is displaced by PI as PI has a stronger affinity constant (3.7×10^5 M) towards nucleic acid than SYTO9 (1.8×10^5 M) [42].

Membrane potential

There is an electric potential across the bacterial membrane that is called the membrane potential; this is due to the K+, Na+, Cl− gradient that is maintained for active transport in viable bacteria. The increase of the membrane potential is termed hyperpolarization and the decrease is termed depolarization. In the event of cell membrane rupture due to stress, the membrane potential reduces to zero and there is a free flow of inorganic ions across the membrane [43]. The membrane potential maintains proton motive force, which in turn plays a role in ATP generation, chemotaxis and other important cellular mechanisms [44]. Carbocyanine dyes such as 3,30-dihexyloxacarbocyanine iodide [DiOC6 (3)] and rhodamine (rh123) are cationic lipophilic dyes that bind to the inner membrane of bacteria that are negatively charged, whereas oxonol dyes such as bis-(1,3-dibutylbarbituric acid) and trimethine oxonol [DiBAC4(3)], which are lipophilic and anionic cannot enter cells with a membrane potential and can only be accumulated in dead cells whose membrane potential has decreased (depolarization) [45]. A combination of these dyes can be used in flow cytometry to determine viable and dead cells.

Esterase substrates

Esterase substrates diffuse into cells because of their neutral charge and are converted into fluorescent end products by the action of intracellular esterases. Therefore viable cells with the ability to produce esterase can convert the susbtrate into fluorescence-emitting products, whereas in dead cells it remains unhydrolyzed and inactive. [46]. This method

Table 2. Dyes used to determine viability and their mechanism of action

Name	Structure	Mechanism of action	Reference
Cyanine dyes		Cyanine dyes belong to polymethine groups and are non-fluorescent dyes that show fluorescence after binding to nucleic acids. They are impermeant to living cells and stain cells with compromised membrane and dead cells	[181, 182]
Calcein		Calcein AM (acetoxymethyl), which is non-fluorescent, converts to a fluorescent calcein due to hydrolysis by esterases present inside cells. It is a cell-permeant dye and stains live cells but is not retained in dead or compromised cells	[183]
Fluorescein diacetate		FDA is a cell-permeant dye that is converted into non-fluorescent fluorescein by the action of intracellular hydrolysis by cells. This helps to determine enzymatic activity as well as membrane integrity during viability detection	[48]
Rhodamine dyes		Rhodamine is a cationic lipophilic dye and is cell-permeable and taken up by cells with active transmembranal potential. Rhodamine 123 and rhodamine B are the commonly used rhodamine dyes in viability studies	[184]
Propidium Iodide		PI is a red fluorescent stain that binds with DNA and its fluorescence is enhanced after binding. PI is used to stain dead or compromised cells as it only enters cells with compromised membranes and hence cannot be taken up by live cells. PI is generally used in combination with another dye that stains viable cells for dual staining	[42]
Ethidium monoazide		EMA is a DNA intercalating agent that binds to the DNA of compromised or dead cells and upon photolysis inhibits PCR amplification of DNA from dead or compromised cells. It is used in combination with PCR-based methods to amplify the DNA of live cells in a technique known as viability PCR	[185]

Table 2. cont.

Name	Structure	Mechanism of action	Reference
Propidium monoazide		PMA similar to EMA is also a DNA-binding photoreactive dye that suppresses the amplification of DNA from dead cells during PCR amplification	[185]
Tetrazolium salts		Tetrazolium salts are heterocyclic compounds that can permeate into intact viable cells and are converted into water-soluble formazan, which is coloured and can be quantified. Tetrazolium salts are reduced within the cell by the oxidoreductases and dehydrogenases present in viable cells	[186]
Resazurin		Resazurin is a redox indicator and a blue non-fluorescent dye. Resazurin is reduced to fluorescent resorufin, which is pink, by the oxidoreductases present in viable cells and hence is used as a vital dye	[187]

indicates enzymatic activity and the membrane integrity of cells. Calcein, fluorescein diacetate and carboxyfluorescein diacetate are some of the commercially available esterase susbstrates used in viability studies. Esterase substrates such as calcein AM (an acetoxymethyl derivative of calcein) converts to a fluorescent end product in the event of acetoxymethyl ester hydrolysis by intracellular esterases [47]. Similarly, fluorescein diacetate and carboxyfluorescein diacetate also convert to fluorescein in the presence of intracellular esterases and are retained within the cell and are visualized by flow cytometry [48].

Viability PCR

PCR-based methods are ideal for the detection and quantification of specific micro-organisms in food, clinical samples and environmental samples. PCR-based methods are rapid, sensitive and robust, but the inability to differentiate between viable and non-viable microbes leads to the overestimation of or false positives for the microbe of interest in samples. [49]. Combining PCR with DNA intercalating dyes has been proven to be efficient in determining viability and has revolutionized microbial detection by overcoming the drawbacks of PCR methods alone. The DNA intercalating dyes only penetrate bacterial cells that have compromised cell membrane and interfere with the DNA amplification of non-viable cells during PCR, hence viability PCR (vPCR) determines the viability of cells based on membrane integrity [Fig. 1] [50]. vPCR was introduced with ethidium monoazide (EMA) being used as the DNA intercalating dye by Nogva *et al.* [51]. A second-generation alternative DNA intercalating dye, propidium monoazide, (PMA) was used with PCR to detect viability and its efficacy was proven by Nocker *et al.* [52].

The cell membrane is a barrier to DNA intercalating dyes in viable cells, whereas they can enter membrane-compromised non-viable cells. DNA intercalating dyes possess an azido group that converts to a reactive nitrene radical on photolysis. The nitrene group cross-links with the DNA of the membrane-compromised cells, inducing structural change in the nucleotide angle, and because of this the DNA polymerase does not bind to the DNA, resulting in there being no elongation, which leads to signal reduction [53]. Furthermore, the DNA cross-linkage with the nitrene group causes it to be insoluble in water and it is eliminated in the DNA extraction steps as a part of the cell debris [54]. The remaining unbound intercalating dye is inactivated by reaction with water molecules, forming hydroxylamine, and is unable to further bind to DNA [52]. Consequently the viable cells, which do not allow intercalating dye to penetrate because of their intact membranes, are unaffected by inactivated dye after cell lysis during the DNA extraction process [55].

The use of EMA has been shown to lead to the loss of DNA from live cells as well during the DNA extraction process. Ethidium bromide, which is structurally similar to EMA, is taken up by viable cells [50]. EMA having a single positive charge is considered to be the reason for its easy penetration into all cells [41]. EMA can permeate and causes loss of DNA in the live cells of bacterial species such as *Anoxybacillus flavithermus*, *Bacillus licheniformis* and *Geobacillus stearothermophilus*, which are common milk powder

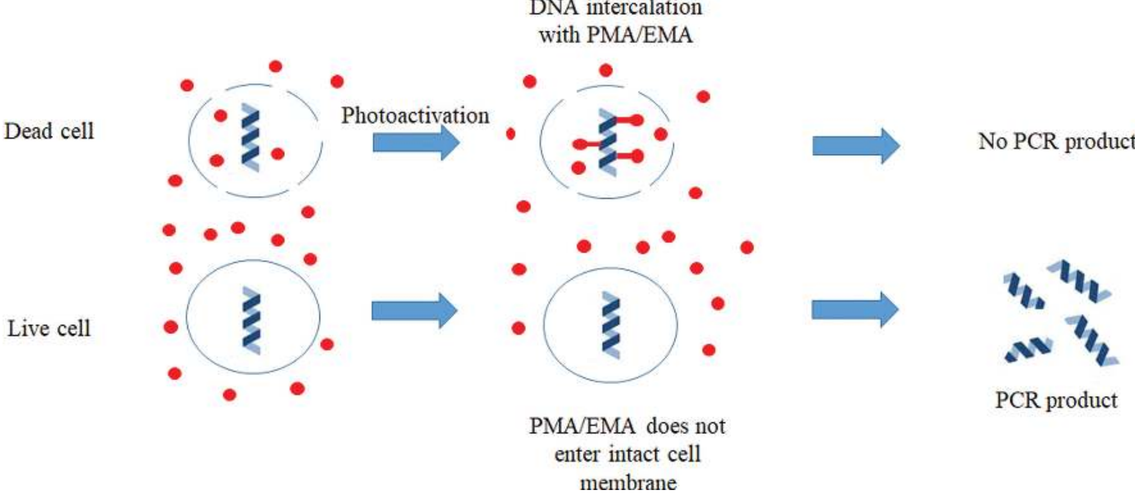

Fig. 1. Mechanism involved in vPCR when using the DNA intercalating dyes PMA/EMA. The dye enters compromised/dead cells, binds covalently to the DNA upon phtoactivation and stops the amplification of DNA from dead cells.

contaminants [56]. EMA is also cytotoxic towards the viable cells of bacterial species such as *Staphylococcus aureus*, *L. monocytogenes*, *Micrococcus luteus*, *Mycobacterium avium* and *Streptococcus sobrinus* [55]. However, *Pseudomonas syringae* species have shown EMA resistance and stain moderately with EMA, leading to DNA loss during extraction. The varying ability of EMA to permeate through the bacterial membranes of different species limits its application in viability determination [55].

PMA, analogously to propidium iodide (PI), with an azide group added to phenanthridine ring and with a double positive charge, is considered to be a second-generation DNA intercalating dye. Like propidium iodide, PMA does not enter live cells and is more specific in differentiating viable and non-viable cells [57]. PMA, with a double positive charge, permeates less and does not permeate into slightly compromised cells and requires a higher concentration to attain the same result as EMA, as observed in species such as *Legionella pneumophilia* [58]. Some of the amplification techniques used after PMA/EMA treatment are listed in Table 3.

RNA-based methods

Nucleic acid sequence-based amplification (NASBA)

NASBA is a continuous self-sustained nucleic acid amplification method that was developed in 1991 by J. Compton [59]. NASBA has been applied to study bacterial viability to determine the antibiotic sensitivity of mycobacterial species [60, 61]. NASBA is an RNA amplification process that takes place in isothermal conditions and without any equipment [59]. The short life of mRNA makes it a more appropriate target to represent the living population of microbes in a sample than DNA. Avian myeloblastosis virus (AMV) reverse transcriptase, RNaseH and T7 RNA polymerase are the enzymes used to simultaneously amplify RNA targets, resulting in 10^9-fold amplification [62]. The process involves the use of two primers, one of which is complementary to the target RNA sequence, while the other behaves as a recognition sequence for T7 RNA polymerase. The reaction mixture also contains dNTPs and NTPs. The RNA target is amplified by the AMV reverse transcriptase enzyme with the help of a primer targeting the RNA sequence to form cDNA [63]. The RNaseH digests the remaining RNA and the second primer binds to the cDNA and converts it into double-stranded DNA using AMV reverse transcriptase, which acts as a transcriptionally active promoter [64]. T7 RNA polymerase then transcribes many copies of target RNA to form dsDNA and the cyclic process continues [59]. The process is carried out at 41 °C as the DNA remains stable and does not denature, which results in the amplification of DNA instead of target RNA [Fig. 2] [63]. DNA does not interfere in the cycle and there is no requirement to use an RNA extraction procedure or DNase to reduce DNA contamination, as in the case of RT-PCR [1]. Many studies have used NASBA to determine the viability of pathogens, such as *Haemophilus influenzae*, *Neisseria meningitidis* and *Streptococcus pneumoniae*, which are associated with bacterial meningitis [65], *V. cholerae* in water sources [66], *E. coli* in drinking water [67] and *Salmonella* spp. in food samples [68]. Recent studies have explored the use of NASBA as a point-of-care (POC) system for the rapid and easy determination of viability to measure antimicrobial resistance, as it eliminates the need for a thermal cycler [69]. However, RNA stability may be compromised during sample collection and storage. This drawback can be overcome by adding RNase inhibitors such as guanidine thiocyanate to ensure the preservation of RNA integrity [70].

Molecular viability test (MVT)

Due to its shorter average half-life, mRNA has been used to detect bacterial metabolic responses and viability [71].

Table 3. Different amplification techniques used after PMA/EMA treatment

Serial no.	Technique used	Organism detected	Sample studied	Reference
1	qPCR	*S. enteritidis*	NA*	[188]
		L. pneumophila, Salmonella typhimurium		[185]
2	Multiplex PCR	*Aeromonas, C. jejuni, C. coli, Salmonella, Shigella*, enteroinvasive *E. coli* (EIEC), *Vibrio*, *Yersinia*, *L. pneumophila, S. typhimurium* and *S. aureus*	Faecal sample and environmental water sample	[189] [190]
3	Microarray	Different molecular operational taxonomic unit (MOTU) corresponding to different phyla detected	Clean room facilities housing spacecraft hardware	[191]
4	DGGE	*Legionella* species	Aquatic environment	[192]
5	454 pyrosequencing	*Cyanobacteria* and *Cryomorphaceae*	Sea and canal water	[193]
6	Ion torrent sequencing	Microbial community analysis	Water sample	[194]
7	Loop-mediated isothermal amplification	*Salmonella* strains	NA*	[195]
8	Metagenomic library construction	Eukaryotes, prokaryotes and viruses	Cleanroom environment	[196]

*Not available.

However, because of its short life span and unstable nature it is a challenge to use mRNA as a marker to determine viability. rRNA has a half-life of days, which is much longer than mRNA and accounts for 90 % of the total cellular RNA, hence it allows better viability determination in microbes [72–74]. However, rRNA has been reported to persist in dead bacterial cells [75, 76]. rRNA precursors called pre-rRNA have been been reported as a target to detect viability in several studies [75–77]. Mature rRNA is formed by enzymatic removal of leader and tail fragments of pre-rRNA [57] and makes up more than 25 % of the total rRNA of the cell in growing bacteria [78]. Pre-rRNA is synthesized in bacteria immediately in response to nutritional stimulus and this synthesis stops when growth slows and the cells become non-viable [79]. This makes pre-rRNA the ideal target for viability detection using RT-PCR [Fig. 3]. The sensitivity of MVT is high due to the release many copies of pre-rRNA after cell lysis [57]. Thus MVT uses the ability to synthesize macromolecule in the presence of nutrition [80]. Cells that can catalyze the process of RNA synthesis and also possess membrane integrity can respond to the nutritional stimulus by producing pre-rRNA [57]. MVT has been used to detect the pre-rRNA upshift in viable bacteria in different environments, such as water sources [75], human serum [76], and milk [80]. MVT has also been proven to be effective in determining the viable cells of bacterial species such as *E. coli*, *A. hydrophila*, *Enterococcus faecalis* [77], *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *S. aureus* and *Mycobacterium tuberculosis* [76]. However, MVT cannot be applied to viruses due to their lack of ribosomes.

Cellular and metabolic properties

Adenosine triphosphate (ATP)

ATP is the energy currency in all living organisms and so is an excellent biomarker for viability microbes, as ATP synthesis is arrested immediately after cell death [81]. ATP concentration within a cell varies depending on growth conditions and under standard growth conditions intracellular ATP concentration can be compared to the cell concentration in suspension. Luciferin–luciferase, which is responsible for bioluminescence in fireflies, influenced the development of the ATP detection system [82].

$$\text{D luciferin}+O_2+\text{ATP}+Mg^{+2}\xrightarrow{\text{firefly luciferase}}\text{oxyluciferin}+\text{AMP}+\text{PPi}+\text{light}.$$

The light intensity correlates directly with the ATP concentration and can therefore be interpreted as the presence of viable microbes. ATP has been used as a biomarker to detect microbial contamination in various environments, such as clean room facilities [83], simulated Martian conditions [84], hospital water sources [85], hospital surfaces and instruments [86, 87], aquatic environments [88] and soil [89].

Exogenous ATP is also measured in a cell suspension using this method, but this can overestimate the viable count and to overcome this drawback the removal of extracellular ATP enzymatically is necessary. Extracellular ATP can be removed by (i) filtration of the cell suspension, or (ii) enzymatic hydrolysis of extacellular ATP using enzymes such as apyrase, which is isolated from potato tubers and converts ATP to ADP, which does not act as a substrate for luciferase. Apyrase does not enter into the cell membrane, and intracellular ATP is not degraded and can be estimated by cell disruption to release intracellular ATP [90]. After the

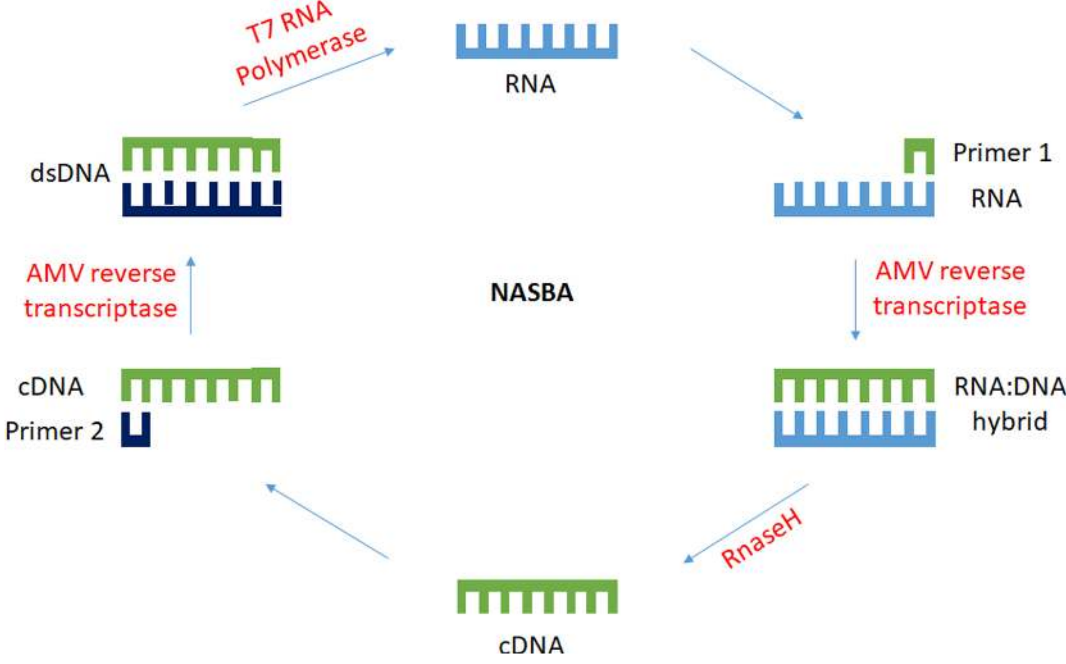

Fig. 2. Schematic representation of the process involved in NASBA using the enzymes T7 RNA polymerase, AMV reverse transcriptase and Rnase H.

removal of exogenous ATP, the cell is lysed and intracellular ATP is measured by bioluminescence assay [85]. Non-microbial sources of ATP also interfere with ATP measurement and may lead to false-positive results.

Respiration

Using tetrazolium salts to detect respiration

The dehydrogenase enzyme present in the electron transport system is used as a measure of respiratory activity of microbial cells. Tetrazolium salts are reduced by the dehydrogenase enzyme and converted into formazan, which is a measure of the total respiratory activity of the cell [Fig. 4]. The quantity of formazan produced is the measure of respiratory activity and is measured colorimetrically. 2,3,5-triphenyl tetrazolium chloride (TTC) [91], 2-(4-iodophenyl)−3-(4-nitro-phenyl)−5-phenyl tetrazolium chloride (INT) [92], 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) [93] and 3′-[1-(phenylamino)-carbonyl]−3,4- tetrazolium]-bis (4-methoxy-6-nitro) benzenesulfonic acid hydrate (XTT), [94] are some of the tetrazolium salts in wide use for the measurement of microbial viability.

Resazurin

Resazurin is an oxidation reduction indicator that is purple in colour and turns pink due to cellular oxidoreductases [Fig. 4]. Resazurin is itself a weakly fluorescent dye that is reduced to a highly fluorescent resorufin. It is an oxidation reduction indicator for aerobic as well as anaerobic respiration. Resaruzin was first reported for the detection of contamination of milk by Pesch and Simmert in 1929 [95]. Resazurin has been used to determine microbial viability for the determination of the minimum inhibitory concentration (MIC) of antibiotics [96], antimicrobial susceptibility testing [97] and drug-resistant bacterial detection [98]. Recent advances include resazurin-amplified picoarray detection to quantify *E. coli* and *S. aureus*. Using this technique, single bacteria can be detected by fluorescence using resazurin to determine the microcolonies formed by single colonies entrapped in picochambers [99].

Direct viable count (DVC)

The DVC method was first reported by Kogure *et al.* in context of the quantification of viable bacteria in the marine environment through incubating samples with nalidixic acid (antimicrobial agent) and yeast extract (nutrient source) [100]. Nalidixic acid inhibits DNA synthesis and the cells continue to use the nutrients to become elongated/fattened.[101]. These cells are easily visualized by the microscopic method and fluorescent microscopy. However, when DVC is applied to enumerate mixed microbial communities, some bacteria may be resistant to the antimicrobial used. To overcome this drawback, an antibiotic cocktail is used and its effectiveness has been proven [102]. Another drawback is the difficulty of differentiating between elongated and fattened cells and cells that have not elongated. The fattened and elongated cells could be missed during the count, as they may be smaller than the average population size in a mixed microbial community. To address this drawback, glycine was used to induce spheroplast in viable cells and this was named the quantitative DVC (qDVC) procedure [101].

Glycine inhibits bacterial growth by disrupting peptidoglycan synthesis, leading to loose cell walls. The viable cells

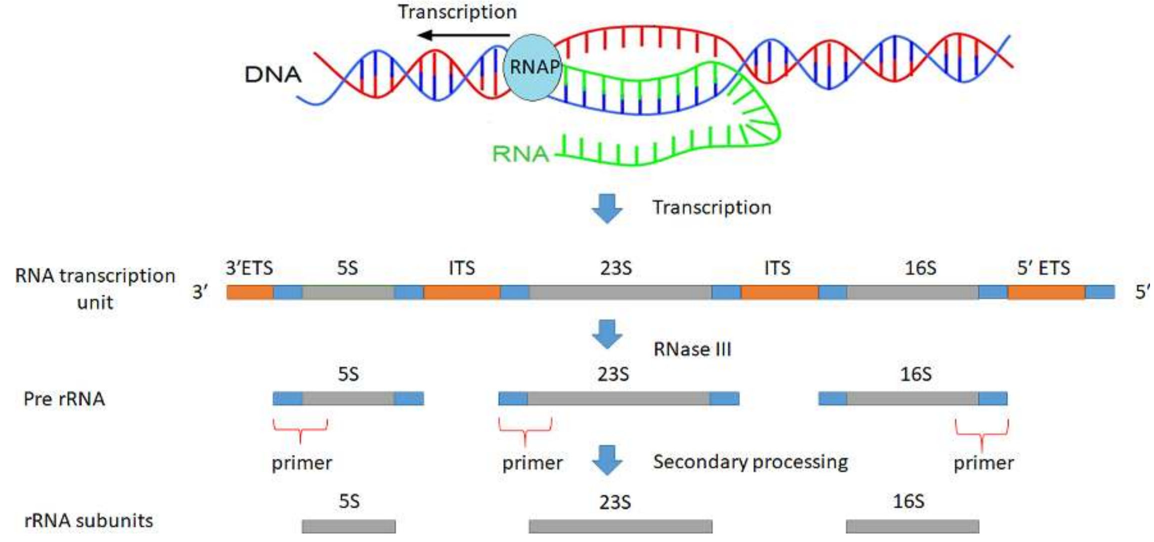

Fig. 3. The illustration describes rRNA synthesis in bacteria . RNA polymerase produces a 30S RNA transcript that is further converted to pre-rRNA subunits with leader and tail sequences that are then removed by endonucleolytic activity to produce mature rRNA. Primers can be designed to recognize pre-rRNA sequences, which can be amplified by RT-qPCR to measure pre-rRNA upshift in response to nutritional stimulus, which is a sign of bacterial viability.

become swollen with loose cell walls, forming unstable spheroplasts, and are easily lysed by freeze/thaw treatment [101]. The elongated/fattened cells are metabolically active and capable of growth. The remaining cells that do not respond to the substrates can be stained and visualized. The number of viable cells is obtained by subtracting the stained cells that remain after DVC treatment and lysing from the total number of cells [103]. qDVC can be used to detect viable cells in mixed bacterial environments with bacterial cells of different sizes, as it is independent of bacterial morphology. DVC procedures have been widely applied in studying the marine environment. They have also been used to assess VBNC *Listeria monocytogenes* contamination in potable water [104]. Further, DVC has been applied to enumerate water-borne bacteria and their viability [105]. Recent studies have used DVC in combination with fluorescent *in situ* hybridization for the detection of bacterial contamination [106]. In one such study, Tirodimos *et al*. described the detection of *Helicobacter pylori* in river water using antibiotic novobiocin, with the resulting swollen cells being used as rRNA targets for a hybridization probe, as intracellular rRNA is increased due to nutritional stimulus [107].

Heat flow

All physiochemical activity of microbes is accompanied by heat flow, which is a biomarker for the viability of cells. The thermal effect of physiochemical activity can be measured by isothermal microcalorimetry (IMC) [108]. Microcalorimeters measure the heat flow between the reaction vessel containing the sample and the heat sink, with these being connected by a thermophile [109]. Commercial isothermal microcalorimeters can detect heat produced of the order of 0.2 µW. Heat production by a single *E. coli* cell has been estimated to be between 1.4–3.5 pW cell^{-1}. [108]. Considering an average of 2 pW cell^{-1}, a value of around 100 000 is required to produce a notable signal that can be picked up by a commercial microcalorimeter. A concentration of about 2.5×10^4–1×10^5 bacteria ml^{-1} is the detectable limit for isothermal microcalorimeters, making them more sensitive than spectrophotometers [109]. The heat produced by bacteria in tropical soils as measured by IMC was correlated to viable counts of bacteria measured using plate counting methods [110]. IMC has been used to detect bacterial contamination and infections such as bacterial contamination of donated blood platelets [111]. Mixed communities of bacteria were analysed in marine sediments efficiently using IMC [112]. IMC has also been used to detect the MIC and efficiency of various antimicrobial compounds, such as selenium compounds, against *S. aureus* [113], cephalopsorins against *E. coli* [114], and also various antibiotics against *E. coli* and *S. aureus* [115]. The application of IMC for rapid pathogen detection in urine has been carried out to determine four pathogens simultaneously, as the heat flow curves are unique for each species, independent of the initial concentration of pathogen in the sample [116]. IMC is convenient for use in combination with any quality control and downstream analysis, as it is a non-destructive technique and does not require any treatment with dyes or other compounds [41]. IMC allows for real-time microbial viability studies to determine the effect of antimicrobials and help in the development of new drugs [117]. However, IMC measures the net signal of all the chemical and physical processes occurring inside the ampoule containing the sample, and unknown phenomena may produce heat that is accounted for in the net signal, and simultaneous exothermic and endothermic reactions may lead to incorrect signals [118].

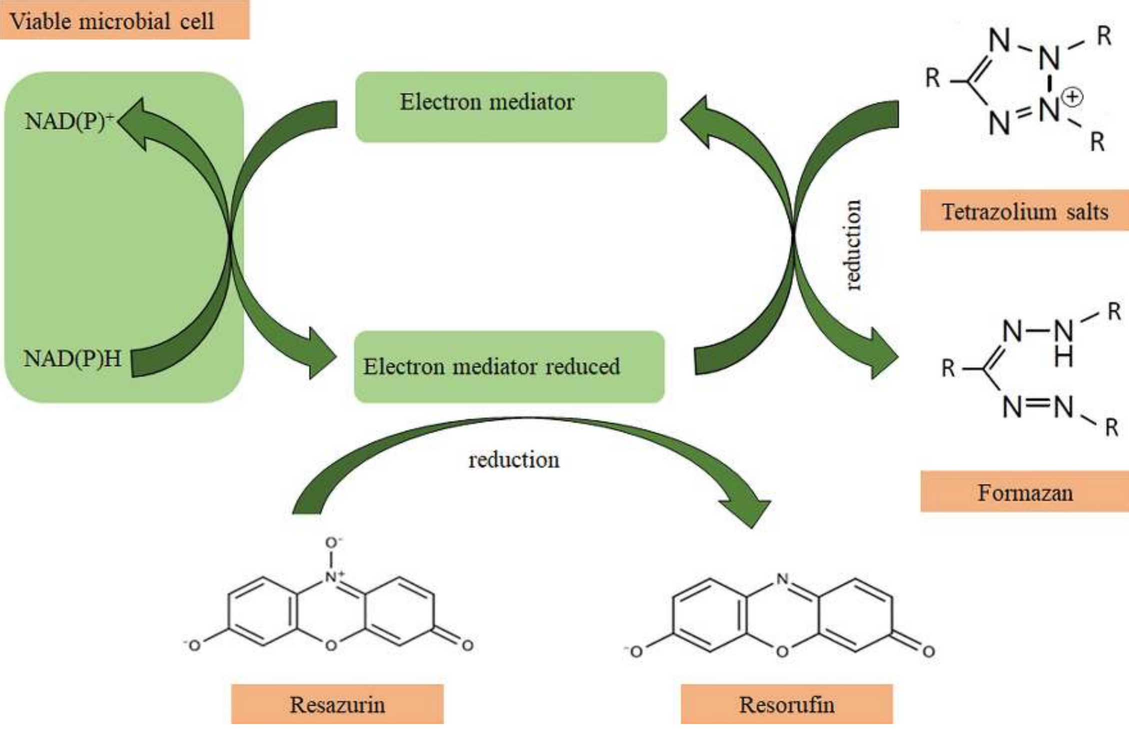

Fig. 4. Oxidoreductases and dehydrogenases present in viable cells reduce tetrazolium salt to water-soluble formazan and resazurin to fluorescent resorufin, which is detected by a colour change and is a measure of the respiratory activity of viable cells.

Stable isotope probing (SIP)

SIP involves the utilization of heavy isotopes by microbes and the incorporation of these isotopes in their DNA. ^{13}C, ^{15}N and $H_2^{18}O$ are some of the isotopically labelled substrates that are metabolized by microbes [119]. Only metabolically active microbes can metabolize and incorporate labelled isoptopes and can be separated from unlabelled microbes. The unlabelled cells essentially include non-viable microbes that are unable to metabolize the isotopes. DNA SIP gives the representation of the replicating cells present in the sample [120]. Labelled DNA can be separated from non-labelled DNA by cesium chloride density gradient with ethidium bromide [121]. ^{13}C- DNA is separated from ^{12}C-DNA and is retrieved. The fraction containing ^{13}C- DNA is representative of the microbes that are metabolically active and are able to incorporate the labelled isotopes [122]. Cell division is essential to incorporate labelled isotopes into DNA and may require a longer incubation time, especially among slow-growing microbes. To overcome these limitations, RNA SIP is better and more suitable for the detection of metabolically active cells. RNA has a faster turnover rate, easily incorporates isotopes and reduces the incubation time [119]. RNA SIP represents the transcriptional activity of the cells by incorporating isotopes [123]. Labelled DNA/RNA detected by SIP is later characterized by gene analysis through fingerprinting analysis or 16S rDNA sequencing approaches. With recent advances in sequencing technology we can obtain the metagenomes or metatranscriptomes of labelled microbes detected by SIP [124]

FUTURE PROSPECTS

Isothermal nucleic acid amplification

Isothermal reactions must take place at constant and low temperature to carry out amplification. Nucleic acid amplification methods such as PCR, although widely used, require a thermal cycler, which is expensive and is not portable. This has motivated research into alternative amplification methods that avoid the use of thermal cyclers to reduce the expense and increase the ease of the process [9]. Conversely, isothermal amplification processes reduce the need for thermal control and power consumption. Some of the isothermal nucleic acid amplification techniques used in viable pathogen detection are listed in Table 4.

Biosensors

The use of biosensors in pathogen detection involves the use of a biomarker that is specific to the pathogen and interacts with a biological receptor (monoclonal antibody, RNA, DNA, glycan, enzyme, whole cell). A transducer helps convert the probe–target interaction into a signal and a data output system to analyse the data. Based on the type of probe and transducer used, different types of biosensor have been used to detect pathogens, for example optical and electrochemical biosensors were used to detect *E.coli* O157:H7

Table 4. Isothermal nucleic acid amplification techniques used in pathogen detection

Serial no.	Technique used	Species detected	Reference
1	Nucleic acid sequence-based amplification	*V. cholerae*	[66]
		H. influenzae, N. meningitidis	[65]
2	Loop-mediated isothermal amplification	*E. coli*	[197]
			[198]
3	Helicase-dependent amplification	*Trichomonas vaginalis*	[199]
		E. coli	[200]
4	Strand displacement amplification	*V. cholerae*	[201]
5	Rolling-circle amplification	*M. tuberculosis*	[202]
		V. cholerae	[203]
6	Recombinase polymerase amplification	*E. coli, Klebsiella pneumoniae, Proteus mirabilis, P. aeruginosa* and *E. faecalis*	[204]

[125], piezoelectric and magnetic biosensors were used to detect *E. coli* [126, 127] and immunosensors were used to detect *E.coli* O157:H7 [128].

Microarrays

Microarrays consist of capture probes immobilized onto solid surfaces by specific surface chemistry. The immobilization of species-specific DNA probes on the surface of a transducer helps to hybridize target DNA with the DNA probe. After washing unbound sample DNA, the resulting stable double-stranded DNA are detected using optical signals by a prelabelled target sequence. A pattern of fluorescence will appear on the array. Microarray technology has been used to determine bacterial contamination by pathogens such as *E.coli, Salmonella* species, *S. aureus* and *C. jejuni* from different sources [129–132]. Common food-borne and water-borne pathogens, such as *Shigella* species, *Salmonella* species, *L. monocytogenes* and *E. coli* O157:H7 have been detected simultaneously in a complex food matrix using a microplate chip in which each microarray was integrated into each well of the microplate [133, 134]. Microarrays have also been used efficiently for the determination of pathogens in complex human samples such as cerebrospinal fluid for the detection of central nervous system infections to aid diagnosis and treatment [135]. Microarrays have proven to be valuable in pathogen determination due to their superior specificity and sensitivity in various sample matrices, and because they have detection limits of as low as 10 c.f.u. ml^{-1}.

Microfluidics

Biological nano- and microelectromechanical systems incorporate many laboratory processes on a chip for the rapid and convenient detection of pathogens using semiconductor technology. Microfluidic chips have been proven to be a promising tool in pathogen detection due to their flexibility for automation, miniaturization and multiplexing [136]. Several microfluidic systems fabricated from nanomaterials have been developed for DNA extraction [137, 138] as well as nucleic acid amplification [139]. Such microfluidic devices have been used to extract DNA and for PCR reaction to detect *E. coli, Streptococcus mutans* and *Staphylococcus epidermidis* [140]. Microfluidic chips have recently been fabricated to carry out loop-mediated isothermal amplification (LAMP) for the rapid detection of multiple pathogens causing pneumonia, which can be carried out in 90 min to detect an abundance of nucleic acid of as low as 10 copies [141]. Microfluidic devices combining LAMP and gold nanoparticles have also been developed for the detection of *Salmonella* spp. in food samples, with a limit of detection of as low as 10 c.f.u. ml^{-1} [142]. Further advances in fabrication methods, miniaturization and data analysis could lead to a miniaturized standalone laboratory technique, enabling its use in low-resource settings for pathogen detection. Three-dimensional printing of microfluidic systems could further reduce the costs and production time [143].

DISCUSSION

Culture-based diagnosis of pathogens has many lacunae that can be addressed using alternative molecular methods that are more reliable and less time-consuming. However, the major difficulty in pathogen detection remain distinguishing between viable and dead cells, and failure to do this accurately results in overestimation or underestimation, leading to the risk of contamination.

There are various methods to determine the viability of microbial communities, but much validation is required when these methods are employed. These methods need to be developed further for their application to determine the viability of different microbes in different environmental conditions. The practicality of the assay and the interpretation of results are important factors when considering different techniques to apply to determine viability [41].

Membrane integrity is compromised when electroporation is used to allow the entry of DNA into cells. A study in which the proton motive force of *Camplyobacter* was abolished showed the entry of EtBr into bacteria, which is a marker for dead cells and compromises the use of membrane integrity as a marker for viability [144]. The use of nucleic acid-based determination of viability is highly dependent on environmental conditions, which may influence viability determination [1]. Several factors are important and influence the application of viability staining using PI and SYTO9, such as the bleaching effect of SYTO9, the binding affinities of SYTO9 and background fluorescence

[42]. Loss of DNA has been reported due to the use of EMA for viability determination and results in the underrepresentation of viability [54], whereas reports have shown that PMA does not completely eliminate signal from dead cells, leading to false positives [2]. Bacterial inactivation by UV, solar disinfection, low-temperature pasteurization and antibiotics do not cause membrane disintegration, and because of this membrane integrity cannot be used to establish viability, hence the manner in which cell death occurs plays an important role in deciding which method of viability determination should be employed [57]. Due to the diversity of cell types, it is not feasible to apply a single universal method or technique to determine viability; using several viability factors will give a better representation of viable cells [145]. Each method has its limitations and may cause species biases and careful consideration of which method should be applied is important.

Limit of detection (LOD) is another factor that influences the selection of viability determination methods. An enrichment step is required during pathogen detection, as the LOD is high for most detection methods. To overcome this issue, immunomagnetic separation (IMS) can be used as a preparatory step. The IMS method is widely used for the selective concentration and isolation of target organisms in a complex matrix. It decreases the time required for detection, as there is no need for an enrichment step for pathogen detection [146]. Further, IMS improves pathogen detection by removing signal from inhibitory agents present in the sample matrix [147]. IMS involves the coating of antibodies or ligands targeting pathogens on super paramagnetic beads, thus forming immunomagnetic beads that target bacteria in a complex matrix, forming a microbe–bead complex [148]. The microbe–bead complex can be concentrated from the matrix by applying an external magnetic field. IMS has been used in combination with PCR [149, 150], flow cytometry with vital stains [150, 151], bioluminescence [152], stable isotope probing [153] and enzyme-linked immunosorbent assay (ELISA) [154], and can be explored as a enrichment step with other viability determination methods.

CONCLUSION

The development of strategies and systems to obtain rapid and efficient detection of pathogenic contamination and reduce the proliferation of pathogens are of the utmost importance to prevent health hazards. Detection methods for contamination must be rapid, cost-effective, versatile and able to test large numbers of many different analytes from various sample matrices. There are many detection methods, however, and methods can be selected based on the type of sample, matrix characteristics, and the equipment and chemicals required, as well as technical proficiency. Further validation and standardization of these methods is required to improve their sensitivity, specificity and repeatability.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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