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Derleme/ Review

Novel Approaches for Monitoring Viable Pathogenic Microorganisms in Environmental Samples

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Abstract: In many industries and scientific fields, for example, food industry, medicine, ecological business and agriculture, contamination of products may have terrible consequences in the manner of public health. Therefore, detection and quantification of indicator microorganisms in many environmental sample and industrial products are the keys for counteractive action and identification of problems related to health issues. Also, since viable portion of the pathogenic microorganisms is likely a threat to public health, developing a novel method to quantify only viable cells is imperative. This review considers the traditional methods and the novel molecular developments with their advantages and drawbacks for detection and quantification of not only pathogenic microorganisms but also any living cells in a sample.
Keywords: Live/Dead differentiation, Pathogen, Propidium monoazide, Q-PCR

Çevresel Örneklerde Bulunan Canlı Patojen Mikroorganizmaları Saptama ve Miktarlarını Belirlemede Yeni Yaklaşımlar

Özet: Gıda endüstrisi, çevre endüstrisi, tıp ve tarım gibi pek çok endüstri ve bilimsel alanda ürün kontaminasyonu halk sağlığı açısından çok kötü sonuçlar doğurabilmektedir. Bu nedenle çevresel ve endüstriyel örneklerde indikatör mikroorganizmaların saptanması ve miktarlarının belirlenmesi halk sağlığı ve güvencesi açısından çok büyük önem taşımaktadır. Patojen mikroorganizmaların canlı olanları büyük ölçüde halk sağlığını tehdit ettiği için sadece canlı hücreleri saptayan ve miktarlarını belirleyen yeni tekniklerin geliştirilmesi çok önemlidir. Bu derleme, bir örnek içerisinde bulunan sadece patojen mikroorganizmaları değil aynı zamanda istenilen diğer canlı hücreleri saptamakta ve miktarlarını belirlemekte kullanılan geleneksel yöntemler ile yeni, güncel moleküler gelişmeleri avantaj ve dezavantajları ile birlikte özetlemektedir.

Anahtar kelimeler: Canlı/Ölü hücre ayrımı, Patojen, Propidyum monoazid, Q-PCR

Introduction

The challenges in detection and quantification of pathogenic microorganisms in any area including food safety, clinical research, drug discovery, animal health, biodefense, and wastewater treatment have increased due to the high demand for different types and healthier products. For example, in food industry, foodborne diseases caused by microbial pathogens, include fungi, viruses, parasites and bacteria; have significantly increased over the last few decades (Oliver et al. 2005). Therefore, it is very crucial to examine the food for the presence of pathogens in order to ensure a safe food provision and to minimize the events caused by contamination. In medical industry, detection, identification and quantification of infectious factors in clinical samples are also very vital in order to provide patients with accurate therapeutic agents. Therefore, an ideal monitoring test ought to be sensitive, particular and also fast to improve the patient recovery and have the ability to minimize adverse reactions to treatments. (Fournier et al., 2014). In wastewater treatment system detection and quantification of pathogenic microorganisms are very important issues also. For wastewater treatment processes the main aim is to eliminate unwanted components in wastewater and making them safe for disposal and public use. Biosolids are treated wastewater originated organic debris from domestic wastewater treatment plants available for safe recycling as a soil amendment (Wang et al. 2008). The most common beneficial use of biosolids is land application as fertilizer or soil amendment in agriculture because of their high nitrogen (N) and phosphorous (P) content (Epstein 2003). Although, there is a growing beneficial reuse of biosolids in land application, presence of human, animal, and plant pathogens is a big concern.

Simultaneous detection and quantification of each pathogenic microorganism in any sample is improbable due to the lack of specific identification techniques for all of them. Also, since some of the pathogens in the sample present in low numbers, quantification of them becomes costly, labor intensive and time consuming. Therefore, use of indicator microorganisms which are always present in the sample as a representative group of pathogens increases the feasibility. For example quantification of indicator bacteria such as *E. coli* is the key for avoidance of problems in biosolids related to public health (USEPA 2003).

Viable but Non-Culturable (VBNC) Cells

Although the complete molecular mechanism is not yet revealed, new studies showed many pathogenic bacteria going into a viable but non-culturable (VBNC) state. (Higgins et al. 2007; Higgins et al. 2008; Qi et al. 2007). Bacteria in this state cannot be cultivated on artificial media on which they would normally grow, but are still alive. They have low levels of metabolic activity but they can be cultured upon resuscitation. Most researchers believe that it is a survival strategy in response to stress conditions. The number of species described to enter the VBNC state constantly increases including a large number of human pathogens; *Campylobacter* spp., *E. coli* (including EHEC strains), *Helicobacter pylori, Legionella pneumophila, Listeria monocytogenes, Mycobacterium tuberculosis, Pseudomonas aeruginosa,* several *Salmonella* and *Shigella* spp. and numerous pathogenic *Vibrio* spp. (Oliver 2010).

These bacteria still pose a threat in any industry and therefore, methods to improve the monitoring these cells in a sample must be developed.

Methods for Detection and Quantification of Indicator Organisms in a Sample

Detection and enumeration of viable microorganisms accurately in the presence of dead ones is of vital importance for many practices such as safety of food products, drinking water quality control, and medical diagnosis. The ideal assay must be sensitive, fast and easy to perform. Current methods can be categorized into three divisions: culture-dependent, microscopy and nucleic acid-based methods.

1. Culture-dependent methods

The standard detection method is to use a selective medium for particular indicator bacterial species. However these traditional methods have some drawbacks. In some cases culturing methods are time consuming and expensive. For example, *Campylobacter* needs about two weeks after inoculation for evaluation of the results (Lazcka et al. 2007). Also as mentioned in previous section, many bacteria enter VBNC state and hence cannot be detected with culture dependent techniques.

2. Microscopy

One alternative to the traditional culture methods is to use protocols using microscopy.

2.1. LIVE/DEAD® *Bac*Light™ viability kit

This kit has been widely used for about twenty years. It is used to make a distinction between live and dead cells in a sample based on selective permeability of the cell membrane (Virta et al. 1998). *Bac*Light includes two nucleic acid-binding dyes, namely SYTO 9™ and propidium iodide (PI). SYTO9 fluorescences green and it can enter all cells indicating total cell number. On the other hand, red fluorescing PI enters only cells with injured cellular membranes, and combination of the two dyes gives red fluorescence (Boulos et al. 1999). Therefore, cells with impaired membranes considered dead are observed as red and those with intact membranes, which are considered alive, fluoresce green (Figure 1). However, one drawback of this method is that differentiation of specific bacterial species from other related species is not possible. Also, several studies indicated that using these dyes does not always differentiate between definite "live" and "dead" populations, transitional cases are also observed (Hoefe et al. 2003; Berney et al. 2007).

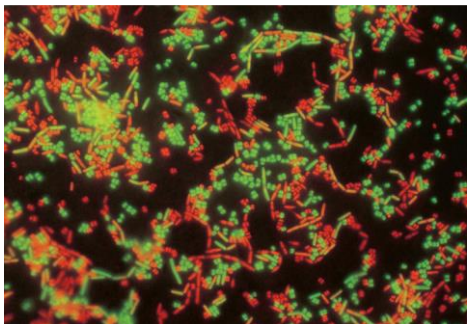

Figure 1. Discrimination of live (green) and dead (red) cells of *Micrococcus luteus* and *Bacillus cereus* combination evaluated using LIVE/DEAD® *Bac*Light™ Bacterial Viability Kits (http://www.thermofisher.com/tr/en/home/technical-resources/research-tools/image-gallery).

2.2. Flow Cytometry

Flow cytometry can be described as automated microscopy analyzing the physical and chemical characteristics of up to thousands of particles per second in a fluid as it passes through the laser (Veal et al. 2000). Although this technique is widely used and has advantages, counting of a small number of bacteria and inability of specifying individual species are the drawbacks (Nocker and Camper 2006).

3. Nucleic acid-based methods

3.1. Fluorescence *In Situ* Hybridization (FISH)

FISH is a hybridization technique that uses fluorescent probes that bind to only very particular sequences of the target nucleic acid with a high specificity. Fluorescence microscopy can be used to visualize the hybridization. It has been used since 1980s in many studies such as species identification, examining the cellular reproduction cycle, chromosomal abnormalities, and visualization of any sequence within a gene or mRNA (Levsky and Singer 2003). Since it is very sensitive technique it can be used for evaluation of indicator organisms in a sample. Preparing DNA probes for the target indicator organism and carrying out FISH enable us to monitor the dispersion of these particular species within a sample. It is very valuable technique for detection but not so easy way for quantification purposes. Since many environmental samples are usually very complex including inorganic aggregates, images may become controversial. Fluorescent probes can bind to those particles and give false positive results (Zhou et al. 2007).

3.2. Real-time Quantitative PCR (Q-PCR)

Q-PCR is a broadly used modern methodology in which products produced during each cycle of the polymerase chain reaction (PCR) are directly proportional to the amount of original template (Ginzinger 2002). There are two strategies in Q-PCR technologies. The first one uses a double-stranded (ds) DNA binding dye. The most common dye is SYBR® Green I. While PCR products increase at each cycle the signal coming from SYBR® Green I also increases. The drawback of this methodology is the lack of specificity since it binds to all dsDNA molecules such as nonspecific PCR products and primer dimer. This may block the accurate quantification of real target sequence.

The second and more reliable strategy is to use sequence specific probes in the protocol. There are several types of specific DNA-based fluorescent reporter probes such as TaqMan® probes, Molecular Beacons™, or Scorpion primers. They are tagged with a fluorophore at one end and a quencher of fluorescence at the opposite end of the probe. Their working principals are slightly different. For example, they can fluoresce either when annealed to the template (as in molecular beacons) or when the probe is broken down by the exonuclease activity of the Taq polymerase. It disrupts the reporter-quencher closeness and thus releases the fluorescence during extension (as in TaqMan probes) (Koch 2004).

3.3. Q-PCR with a Specific Dye Modification

One challenge in molecular biology is to exclude the extracellular DNA and DNA of dead cells during detection and quantification protocols. Recently, new and promising DNA-based strategies have been

introduced. The aim of these methods is to disqualify the DNA molecules coming from dead cells or free environment during DNA extraction or PCR reactions. For this purpose photo-inducible nucleic acid binding dyes have been progressively used in the last decade. Ethidium monoazide (EMA) and propidium monoazide (PMA™) are the most commonly used ones in these alternative strategies (Takahashi et al. 2017; Tantikachornkiat et al. 2016; Bae and Wuertz 2009; Nocker and Camper 2006; Nocker et al. 2006; Wagner et al. 2008).

The key property of these dyes is that they are cell membrane-impermeable. They can enter preferably into the dead cells whose cell membrane is compromised. Among them PMA™ has been shown as a more effective dye distinguishing viable cells from dead ones due to its higher positive charge (Nocker et al. 2006). When it binds to any DNA, DNA is permanently modified through photolysis. As a result, DNA sequences covered by PMA™ molecules cannot be amplified. Consequently, the following PCR amplification produces only results from the viable, intact cells (Figure 2).

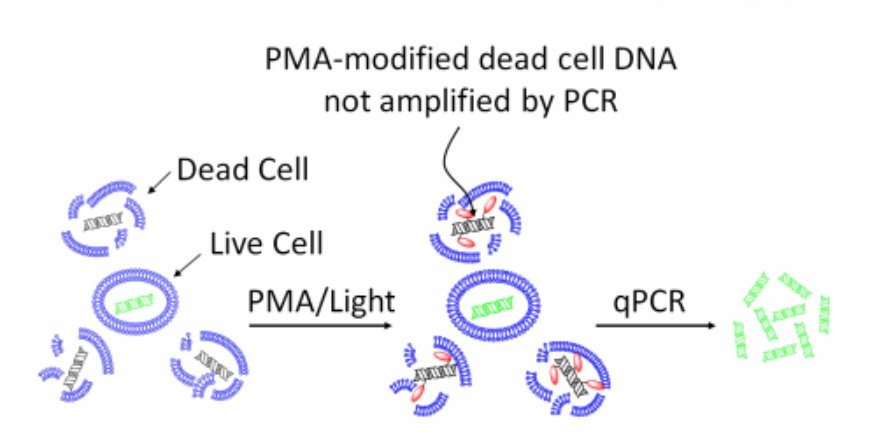

Figure 2. Selective detection of live cells by qPCR with PMA modification (https://biotium.com/product/pmatm-dye-propidium-monoazide/).

Conclusion

Many strategies have been developed for quantification of microorganisms especially bacteria from various samples. Conventional methods are designed for monitoring the culturable species on a suitable medium containing carbon and/or other energy sources. However, there are several obstacles of these traditional culture-based methods. Sometimes they may be time consuming and laborious and all bacterial cells, especially those that are injured or VBNC, cannot be detected. Culture-independent methods have removed these limitations and provided new tools for detection, identification and quantification of the targeted bacteria (Hazen et al. 2013). If the aim is just the detection and identification rather than quantification, there are more alternatives than those mentioned in the previous sections. Traditional PCR and multiplex PCR are widely used nucleic acid amplification methods. Different fingerprinting methods such as Pulsed-Field Gel Electrophoresis (PFGE), Density Gradient Gel Electrophoresis (DGGE), Amplified Fragment Length Polymorphism (AFLP) and Restriction Fragment Length Polymorphism (RFLP) are currently employed to analyze the microbial communities in various habitats (Wagner et al. 2015). However, the fidelity of all these molecular methods is based on extraction of nucleic acids from the accurate sources. Results coming from dead cells or free DNA molecules may give false positive results (Smith and Osborn 2009).

Several, often indefinite, parameters such as dead cells and extracellular DNA handicap the DNA extraction methods. These undesirable factors can cause overestimation of possible health risks since DNA can persist in the environment after cell death (Josephson et al. 1993; Masters et al. 1994). Also it was indicated that mRNA molecules may also present in a detectable form for many hours after cell death (Keer and Birch 2003). The other important need for novel molecular-based methods is the existence of VBNC bacteria. Several studies have demonstrated that VBNC cells of many pathogens continue to produce virulence factors and thus more effective methods of pathogen detection must be employed (Ayrapetyan and Oliver 2016).

In the last years, cell-impermeable nucleic acid binding dyes have been increasingly used before extraction and qPCR to accurately differentiate dead and viable cells. Among them PMA™ is left out by live cells more efficiently than EMA. Due to its positive charge, PMA™ cannot enter cells with a whole, healthy cell membrane, but it selectively penetrates cells with a compromised cellular membrane (Nocker et al. 2006). During intense visible light exposure, dye interacts with dsDNA and forms a covalent bond causing irreversible modification of DNA, which cannot be amplified by PCR. This makes the dye very preferable in the selective evaluation of viable cells. PMA™ has been successfully graded in a variety of studies. These studies include Gram-positive and Gram-negative bacteria (Nocker and Camper 2006, Bae and Wuertz, 2012, Taylor et al., 2014), viruses (Sánchez et al. 2012), fungi (Vesper et al. 2008), spores (Rawsthorne et al. 2009), protozoa (Alonso et al. 2014) and archaea (Heise et al. 2016). Also, it has been applied in complex environmental matrices such as human feces, wastewater treatment plant influent and effluent (Bae and Wuertz 2009, Lin et al. 2011) and biosolids (Taskin et al. 2011).

The existence of dormant cells like VBNC cells in the environment increases the risk to public health. They limit the standard methods for the detection and quantification in a sample. Although researchers have begun to develop new methods, much more work in this area remains to be done. Furthermore, VBNC cells in the environment remain as mystery with respect to scientific knowledge. Therefore, finding methods with improved detection of dormant cells is not only beneficial to public health, but may lead to major discoveries in the field of microbiology.

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