Microbiology Trapped in the 19th Century

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When the science of microbiology was in its early stages of development, scientists used liquid media for the cultivation of microorganisms. For those who were in need of a method to segregate individual types of organisms, the use of liquid media proved to be a significant disadvantage. This was the case for Dr. Robert Koch, who, in 1881, was determined to find an alternative method for his experiments. His laboratory first used aseptically cut slices of potato as a solid culture medium, and later turned to liquid culture supplemented with gelatin, which was subsequently poured into glass plates and allowed to solidify. This technique permitted the scientists to obtain pure cultures of the bacteria that were found to be growing in the form of discreet colonies on the surface of the plates.

Unfortunately, on hot summer days, the gelatin medium would liquefy, rendering it useless for its intended application. Furthermore, this phenomenon was accelerated when certain types of bacteria growing on the surface would produce enzymes capable of digesting the gelatin medium. One of the scientists, Dr. Walther Hesse, was frustrated by these events, and he turned to his wife and laboratory assistant, Angelina for help. Walther recalled that his wife's jellies and puddings remained solid, even in the heat of the day, and when he inquired as to what her secret was, she provided an unusual but simple answer. Angelina was previously made aware of a cooking ingredient called Agar-Agar, which had been used as a gelling agent by Asian chefs for many centuries, and as a result, used the material in many of her recipes. Dr. and Mrs. Hesse discussed the possibility of using Agar-Agar as the basis for a stable, solid microbiological medium, and subsequent experiments showed that this worked magnificently.

This simple kitchen ingredient revolutionized the science of microbiology as it made what had been a difficult task of separating and culturing microorganisms on solid surfaces a routine procedure. Interestingly, more than 125 years later, all microbiology laboratories, in every industry sector, continue to use agar as the most important and widely accepted material for growing microorganisms today. Angelina would be proud...but should we be proud as well?

Although the growth of microbial cells on agar surfaces provides the laboratory with critical information about the amount and the type of organisms that may be present in a sample under evaluation, the time to result is usually longer than what is desired. Days and even weeks may elapse before microbial colonies are visually detected, and in most cases, confluent growth prevents individual organisms from being isolated, necessitating sub-culture onto additional agar media, delaying the time to result even further. Additionally, many laboratories are discovering that microorganisms, when stressed due to nutrient deprivation, or following exposure to sub-lethal concentrations of antimicrobial agents, such as preservatives, disinfectants, heat or decontaminating gases, may not replicate when cultured on artificial media, because the environment is not truly optimal for the resuscitation and subsequent proliferation of organisms that may be present. For these reasons, the modern microbiological laboratory should look toward developing innovative approaches to the detection, quantification and identification of microorganisms. From a quality risk management perspective, this direction is critical for the pharmaceutical and biopharmaceutical industries.

Rapid Methods

Effective monitoring of our manufacturing processes can help to ensure that a state of control is maintained (providing assurance of the continued capability of processes and controls to meet product quality), areas for continual improvement are identified (helping to understand and reduce process variability), process and product understanding is enhanced, and manufacturing agility and efficiencies are realized (by reducing waste and wasteful activities, reduce lead time and increase manufacturing capacity). From a microbiology perspective, we can apply these principles in order to design a process to prevent contamination, investigate ways to correct a contamination event, and assess the potential impact of failing results on the patient.

> **Fortunately, recent advances in alternative microbiological monitoring platforms, such as rapid microbiological methods (RMM), provide the analytical tools necessary to accomplish these tasks.**

An Introduction to Rapid Microbiological Methods

During the past 20 years, the field of alternative and rapid microbiological methods has been gaining momentum as an area of research and application across a number of technology sectors. In fact, much of the development of new systems for the detection and identification of microorganisms has been driven by consumer and patient needs within the food, beverage, environmental and clinical or health care industries. Recent advances in rapid technologies have also encouraged the pharmaceutical and biopharmaceutical industries to validate and implement RMMs in place of their traditional microbiology methods within QC/QA labs and on the manufacturing floor.

Many rapid microbiological method technologies provide more sensitive, accurate, precise, and reproducible test results when compared with conventional, growth-based methods. Furthermore, they may be fully automated, offer increased sample throughput, operate in a continuous data-collecting mode, provide significantly reduced time-to-result (e.g., from days or weeks to hours or minutes), and for some RMM platforms, obtain results in real-time. These methods have also been shown to detect slow-growers and/or viable but non-cultural microorganisms as compared with standard methods used today. Most importantly, a firm that implements a RMM in support of sterile or non-sterile manufacturing processes may realize significant operational efficiencies during the monitoring and controlling of critical process parameters, reducing or eliminating process variability, and reducing the risk to patients. Additional benefits may include the elimination of off-line assays and a reduction in laboratory overhead and headcount, lower inventories (raw material, in-process material, and finished product), a reduction in warehousing space, and a decrease in repeat testing, deviations, out-of-specification investigations, reprocessing or lot rejection.

Rapid Method Scientific Principles

Current rapid method technologies can detect the presence of diverse types of microorganisms or a specific microbial species, enumerate the number of microorganisms present in a sample, and can identify microbial cultures to the genus, species and sub-species levels. The manner in which microorganisms are detected, quantified or identified will be dependent on the specific technology and instrumentation employed.

Growth based methods

Growth-based technologies rely on the measurement of biochemical or physiological parameters that reflect the growth of microorganisms. These types of systems require the organisms in a sample to proliferate, either on a solid or liquid medium, in order to be detected and/or quantified.

Rapid methods that employ the use of growth-based platforms are, for the most part, decreasing the time at which we can detect actively growing microorganisms. Many currently used growth-based systems continue to use conventional liquid or agar media. As a result, the same types of applications that traditional methods are used for can also be applied to growth-based RMMs. Examples include bio-burden testing, Microbial Limits, environmental monitoring, sterility testing, and the identification or presence/absence of microorganisms. Examples of the types of core technology principles that are currently used are based on impedance microbiology, the detection of carbon dioxide (CO_2), the utilization of biochemical and carbohydrate substrates, the use of digital imaging and auto-fluorescence for the rapid detection and counting of micro-colonies, fluorescent staining and enumeration of micro-colonies by laser excitation, and the use of selective media for the rapid detection of specific microorganisms.

Viability methods

Viability-based systems use viability stains and laser excitation for the detection and quantification of microorganisms without the need for cellular growth. Flow cytometry and solid-phase cytometry technologies are examples.

We have come a long way in terms of the detection and quantification of microorganisms. Many in the industry are now using viability-based technologies which can differentiate living cells from dead cells, and can even target specific types of microorganisms using nucleic acid, enzymatic or monoclonal antibody probes. In many cases, direct labeling of individual cells with viability stains or fluorescent markers has been demonstrated with no requirement for cellular growth. And because microbial growth is not required, many organisms that would have a difficult time growing in or on conventional medium, such as spores, stressed and physically-injured cells, fastidious organisms, and viable, but non-culturable isolates, can now be easily and quickly detected and/or enumerated. Since viability labeling of microorganisms can occur within hours or even minutes, near real-time detection and quantitative results may be attained.

Cellular methods

Cellular Component-based RMMs rely on the detection and analysis of specific portions of the microbial cell, including ATP, endotoxin, proteins and surface macromolecules.

Cellular-component based RMMs rely on the analysis of cellular markers or the use of probes that are specific for microbial target sites of interest. Examples include ATP bioluminescence, the detection of endotoxin, and the use of MALDI-TOF mass spectrometry for microbial identification.

ATP Bioluminescence

ATP bioluminescence is the generation of light by a biological process, and is most recognized in the tails of the American firefly *Photinus pyralis*. First discovered in 1947 by William McElroy, he described the ATP bioluminescence reaction in which ATP (Adenosine Triphosphate) is enzymatically consumed to produce light. Specifically, in the presence of the substrate luciferin, the enzyme luciferase will use the energy from ATP to oxidize luciferin and release photons. The photons can then be detected and measured by a luminometer equipped with a photomultiplier tube.

Because all living cells store energy in the form of ATP, this cellular component can be used as a measure of organism viability. Basically, we can capture the microorganisms of interest, release the ATP from within these cells, add the luciferin and luciferase reagents, and measure the amount of bioluminescence generated. The sensitivity for ATP bioluminescence (i.e., how many cells are required to detect a sufficient amount of photons or light) is between 100-1,000 bacterial cells and a single yeast/mold cell. For this reason, when low numbers of bacterial cells are expected in a test sample, an enrichment step in media may be required to allow these cells to multiply and produce a sufficient level ATP for subsequent detection.

A number of ATP bioluminescence systems are currently in use within our industry. Many will provide data in terms of a relative light unit (RLU), which can be correlated with viable cell concentration. In this regard, these systems are primarily used for estimating the cell count based on the specific amount of light that is detected, or for presence/absence testing. The latter can be accomplished by understanding what the lower level of detection is (in terms of RLUs), and if the results are below the level of detection, then no viable organisms are present. This is the basis for the manner in which GSK uses ATP bioluminescence for the early release of a non-sterile prescription nasal spray (i.e., up to four days earlier release than conventional methods).

Another RMM utilizes a more direct enumeration approach by using a membrane filtration technique that employs a hydrophilic filter containing more than 600 compartments separated by hydrophobic partitions. The test sample if filtered through this membrane and individual cells are captured within these compartments. The filter is then transferred onto a suitable agar medium, and the single cells will form microcolonies during a short incubation period. The filter is subsequently treated with an ATP-releasing agent, and the same luciferin and luciferase reagents as described above, and then a special luminometer is used to intensify the bioluminescence from

each microcolony. The bioluminescence signals are then quantified, and a cell count (i.e., the number of microcolonies arising from a single cell) is provided.

ATP bioluminescence is limited by the fact that an organism can contain only a finite amount of ATP (e.g., an average bacterial cell contains 1 attomole of ATP). For this reason, one RMM supplier developed a novel method to amplify the amount of ATP generated in a cell. All living microorganisms contain adenylate kinase (AK), another vital part of energy metabolism. Because AK is an enzyme, rather than a metabolite, it is possible to use AK to generate almost unlimited amounts of its products, including ATP. For example, AK catalyzes the linear amplification of ADP to high levels of ATP. Therefore, if we extract both ATP and AK from a cell, add ADP, luciferin and luciferase, the resulting reaction can produce a 1000-fold increase in ATP, which is detected by the supplier's luminometer.

Today, many companies have employed the use of ATP bioluminescence for presence/absence testing, bioburden estimations, hygiene monitoring and most recently, finished product sterility testing.

Endotoxin Testing

The most widely used methods for endotoxin detection employ Limulus Amebocyte Lysate (LAL), which is isolated from the blood of the horseshoe crab (*Limulus polyphemus*). Many laboratories utilize bench-top instruments for this type of testing, which require samples to be transferred from the manufacturing floor or other locations to the laboratory. Recent advances in endotoxin instrumentation now allow for testing to be performed at the point-of-use or point-of-sampling, with results in as early as 15 minutes. One such LAL-based technology uses a disposable cartridge and hand-held incubating spectrophotometer to allow for a quantitative, kinetic chromogenic method by measuring the color intensity directly related to the endotoxin concentration in a test sample. Each cartridge contains LAL reagent, chromogenic substrate and control standard endotoxin. The sensitivity of this RMM is 0.01-1.0 and 0.05-5.0 EU per mL, and is FDA-approved as an alternative to traditional LAL testing methods for final product release. Many companies are now using this technology for testing pharmaceutical grade water systems (at the point-of-use), raw materials, in-process samples and finished product.

Nucleic Acid methods

Nucleic acid amplification-based technologies employ a variety of scientific principles, including PCR-DNA amplification, RNA-based reverse-transcriptase amplification, 16S rRNA typing, gene sequencing and other novel techniques.

Nucleic acid and gene amplification technologies (also known as genetic or molecular methods) are now recognized as providing superior results when compared with other microbiology methods for particular types of assays. For example, there is a growing trend that more and more pharmaceutical companies are implementing genetic methods for microbial identification and detection applications due to their greater accuracy, speed to result, automation and throughput as compared with classical methods. And the regulatory authorities have also acknowledged the advantages in these types of rapid methods. The FDA Aseptic Processing Guidance recommends

the use of rapid genotypic methods for microbial identification, as these methods have been shown to be more accurate and precise than biochemical and phenotypic techniques, and they are especially valuable for investigations into failures (e.g., sterility test; media fill contamination). Both the European and Japanese Pharmacopeias now recommend PCR for Mycoplasma testing. And the Australian Therapeutic Goods Administration (TGA) requires that a sensitive method of microbial identification, such as molecular typing techniques utilising RNA/DNA homology, is utilized when the identification of an isolate is used to invalidate a sterility test.

Nucleic acid and gene amplification technologies utilize a number of scientific principles, including different types of polymerase chain reaction (PCR), transcription-mediated amplification, 16S rRNA typing (ribotyping), and gene sequencing of specific targets of interest, to name a few. Many of these methods have been designed to rapidly detect the presence of a specific microorganism (e.g., an objectionable or specified “compendial” organism), or generate data that can be used to determine the identification of a recovered microbial isolate, from the Genus level down to the sub-species and/or strain level. Additionally, for systems that utilise amplification platforms, the number of amplification cycles to reach a certain threshold level can now be used to estimate the number of organisms in the original sample. Although there is not enough space to discuss all of the available technologies on this page, an overview of the most commonly used nucleic acid amplification rapid method platforms is provided.

Ribotyping

To maintain correct RNA structure and ribosome function in bacteria, the 16S sequence of rRNA is highly conserved at the Genus and species level. This is one reason why Bergey’s Manual of Systemic Bacteriology now uses 16S rDNA sequences as the standard for taxonomic classification of bacteria. However, the non-conserved fragments within the rRNA operon (the spacer and flanking regions of the 16S sequence) can be used to differentiate strains within a particular species. One RMM technology makes use of the DNA sequences that encode for the rRNA operon for microbial identification and strain differentiation. In this fully-automated system, DNA is extracted from a pure culture of bacteria (e.g., using heat inactivation and lysis agents). The extracted DNA is then cut into smaller fragments using restriction enzymes, such as EcoRI or PvuII. The fragments are then separated according to size by gel electrophoresis and immobilized on a nylon membrane (this is commonly referred to as an automated Southern Blot technique).

The double-stranded DNA is then denatured to single-stranded DNA, and the membrane is subsequently hybridized with a DNA probe (derived from an E. coli rRNA operon). Finally, an antibody-enzyme conjugate is bound to the probe and a chemiluminescent agent is added. Light emitted by the fragments is captured, and the image or banding pattern is compared with patterns stored in the system database. If the pattern is recognized, a bacterial identification is provided. The pattern can also be used to determine if the same strain has been previously observed. This may be helpful when investigating the source of an environmental isolate or a failed microbiology event, such as a positive sterility test or contaminated media fill.

Polymerase Chain Reaction (PCR)

A number of RMM detection and identification systems employ different types of PCR as their underlying core technology. In a classical PCR reaction, DNA is extracted and heated to separate the double strands. DNA primers (short, synthetic sequences) are added, which bind to unique target sequences on the template DNA, if they are present. A heat-stable DNA polymerase, such as Taq DNA polymerase, and nucleotide bases (i.e., A, T, G, C) are then added. The primer is elongated, producing two new complete copies of the template DNA strands. This process is repeated, resulting in millions of copies of the target DNA in a short period of time. Real-time, quantitative PCR measures the DNA amplification reaction as it occurs, while providing an understanding of the amount of target DNA that was in the original sample. In this instance, we can also correlate the number of amplification cycles with an estimation of the number of microorganisms in the sample, in addition to obtaining information about the presence of specific microbial species.

There are a number of commercially available rapid method systems available today that utilize PCR to detect the presence of certain types of microorganisms as well as to estimate viable cell counts. A variety of primers and detection probes also make multiplexing, or the ability to detect more than one DNA target at the same time, a reality. Many of the systems are now semi- or fully-automated, and the types of organisms that can be detected are broad-based, and include bacteria, yeast, mold and Mycoplasma.

Reverse Transcriptase PCR

A modification of the classical PCR reaction utilises RNA as a starting template for the PCR reaction, instead of DNA. Here, the enzyme Reverse Transcriptase (RT) will convert extracted RNA into a complimentary strand of DNA (cDNA). For example, a primer first anneals to the target RNA sequence, if present. Then, RT synthesizes the cDNA. Next, RNAse H removes the remaining single-strand RNA, and a second primer anneals to the cDNA. DNA polymerase will synthesize the second cDNA strand, resulting in double-stranded cDNA, which is then used in the classical PCR reaction. The reason why RT-PCR is so powerful is that RNA is a better marker of cellular viability than DNA, because RNA is not as stable outside of the cell as DNA is. Additionally, there is less risk of detecting (and amplifying) DNA from non-viable cells or residual DNA from the sample and/or work environment. RT-PCR is now being used for the detection of specific types of microorganisms and the estimation of viable cell count.