Validation Study of Rapid Assays of Bioburden, Endotoxins and Other Contamination

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Microbial testing performed in support of pharmaceutical and biopharmaceutical production falls into three main categories: detection (qualitative), enumeration (quantitative), and characterization/identification. Traditional microbiological methods are listed in the compendia and discussed by using the conventional growth-based techniques, which are labor intensive and time consuming. In general, such tests require several days of incubation for microbial contamination (bioburden) to be detected, and therefore management seldom is able to take proactive corrective measures. In addition, microbial growth is limited by the growth medium used and incubation conditions, thus impacting testing sensitivity, accuracy, and reproducibility.

For more than 20 years various technology platforms for rapid microbiological methods (RMMs) have been developed, and many have been readily adopted by the food industry and clinical microbiology laboratories. Their use would certainly offer drug companies faster test turnaround times to accommodate the aggressive deadlines for manufacturing processes and product release. Some rapid methods also offer the possibility for real-time microbial analyses, enabling management to respond to microbial contamination events in a more timely fashion, and can provide cost savings and higher efficiencies in quality control testing laboratories. Despite the many proven business and quality benefits and the fact that the FDA’s initiative to promote the use of process analytical technology (PAT) includes rapid microbial methods, pharmaceutical and biopharmaceutical industries have been somewhat slow to embrace alternative microbial methodologies for several reasons. The major reason is that the bioburden counts detected by the incubation method and rapid assay are greatly divergent.

The use of rapid methods is a dynamic field in applied microbiology and one that has gained increased attention nationally and internationally over time. This topic has been extensively addressed at conferences and in published documents around the world. More recently, the use of alternative methods for control of the microbiological quality of pharmaceutical products and materials used in pharmaceutical production has been addressed by the compendia in an attempt to facilitate implementation of these technologies by pharmaceutical companies. The author presents some of the rapid method technologies under evaluation or in use by pharmaceutical microbiologists and the current status of the implementation of alternative microbial methods.

Key words : Rapid Assay / Bioburden / Endotoxin / Contamination.

INTRODUCTION

FDA Guidance for Industry PAT-A Framework for Innovative Pharmaceutical Development, Manufacturing, and Quality Assurance was issued to encourage pharmaceutical manufacturers to develop and implement effective and efficient innovative approaches in providing quality pharmaceuticals to the public (FDA, 2004a). The linkage of rapid microbiological methods (RMMs) to Process Analytical Technology (PAT) is largely based on real-time release, which is the ability to evaluate and ensure the acceptable quality of the in-process and/or final product on the basis of the collection and analysis...
of in-process data. As stated in the FDA guide, the PAT component of real-time release typically includes a valid combination of assessed material attributes and process controls. Material attributes such as bioburden, endotoxin content, and sterility could be assessed using direct and/or indirect process analytical methods. The combined process measurements and other test data gathered during the manufacturing process could serve as the basis for real-time release of the final product and would demonstrate that each batch conforms to established regulatory quality attributes. The FDA considers real-time release to be comparable with alternative analytical procedures to the compendial microbiological tests for final product release. It is notable that the guidance document stated that the real-time release as defined in this guidance builds on parametric release of terminally heat sterilized drug products. In real-time release, material attributes such as formulation, bioburden, container size, and load pattern, as well as process parameters such as sterilization parameters, are measured and controlled.

In this paper, the author will attempt to define the role of RMM in PAT and discuss the application of RMM to aseptic filling, biopharmaceutical upstream and downstream processing, environmental monitoring and control in clean rooms, next the selection, development, validation, and implementation of RMM for PAT applications, then the industry, regulatory, and compendial guidelines for RMM and finally, regulatory approval of RMM and the future of RMM in pharmaceutical and biopharmaceutical manufacturing.

**TRADITIONAL MICROBIAL TEST METHODS**

Traditional USP microbial testing methods, as referee tests, rely on the growth of microorganisms in culture media for detection, enumeration, and selective isolation. These traditional methods continue to be used because of their long history of use, simplicity, effectiveness, low cost, and suitability for use in all microbiological testing laboratories. However, serious questions can be raised if the continued use of these traditional methods is the right strategy to improve the quality and efficiency in the pharmaceutical industry. Those traditional methods were originally designed for the detection of human pathogens and not for the microbiological quality control of pharmaceutical processes and products. The drivers of the microbial testing should be the critical microbiological quality attributes associated with a specific drug product and the risk assessment of the potential for microbial contamination of that drug product and resulting patient infection. The next few paragraphs will discuss the industry experience with compendial microbial testing.

**Bioburden Testing**

Nonsterile drug substances, pharmaceutical excipients, and drug products are evaluated for bioburden using microbial limit or microbiological examination tests. The traditional test procedures are clearly unsuitable for PAT applications due to their extended incubation times, relative insensitivity, and low precision, and even have limitation as release test methods as they may not detect all objectionable microorganisms that could be present in a nonsterile drug product.

**Sterility Testing**

Sterility testing has traditionally been conducted by inoculating a microbiological broth with an aliquot of the test material and scoring growth by the detection of turbidity. The compendial sterility tests have been harmonized in terms of media, growth-promotion requirements, suitability tests, incubation conditions, number of containers and amounts of material tested, and observation and interpretation of the results. Limited local requirements from the different pharmacopoeias were included in the compendial tests and these were removed in May 2009. The membrane filtration test is the preferred test over the direct inoculation test as it has the capacity to test the entire contents of a product container and inhibitory substances may be rinsed from the membrane. The incubation period for the test is at least 14 days, making it clearly unsuitable for a PAT application.

**Bacterial Endotoxin Testing**

Bacterial endotoxins are pyrogenic materials, for example, lipopolysaccharides, present in the cell wall of gram-negative bacteria. Bacterial endotoxins, if present in injectable products, can lead to dose-related adverse reactions in patients receiving injections ranging from chills to fever to death. A threshold pyrogenic dose is 5 EU per kg of body weight for a parenteral administration. In terms of weight and not potency, this is about 1 ng per kg of body weight for *Escherichia coli* and 50 to 70 ng/kg for *Pseudomonas aeruginosa* in both rabbits and humans. For *E. coli*, this represents some 10,000 whole cells per kg. The *in vivo* rabbit pyrogen test was replaced by the *in vitro Limulus amebocyte lysate* (LAL) endotoxin test in the mid-1970s making the test suitable for both in-process and finished product testing. This test is largely t of responsible for the elimination of pyrogens from parenteral drug products. As different sources of endotoxins have differing potency, the standard was assigned potency in endotoxin units (EU).

The compendial bacterial endotoxin assays and reference standards have been harmonized in terms of test
methods, that is, gel-clot, turbidimetric (end-point and kinetic) and chromogenic (end-point and kinetic) assays, reagents, reference standard, calculation of endotoxin limits for drug products, suitability testing, and assay validation. It should be noted that the gel-clot method is semiquantitative in that it determines the lowest two-content of a product, the referee test is the longer limitation, in the event of a dispute as to the endotoxin \( \text{D-value} \), to the sterilization process would be considered.

Other Testing

Other tests conducted during parenteral manufacturing may not be compendial. A bioburden evaluation of a drug substance, excipient, in-process material, presterile bulk solution, packaging component, or nonsterile drug product is a noncompendial procedure to evaluate the number and type of microorganisms per unit weight, item, or unit surface area of the material. Typically in product development, a bioburden evaluation is a non-Good Manufacturing Practice (non-GMP) screening test that may not be fully validated or have regulatory status as part of a risk assessment undertaken during formulation and manufacturing process development. For example, as part of the sterilization process development, the numbers, cellular morphology, cell size, staining reactions, and spore-forming capabilities of the predominant microbial population associated with the material would be determined to establish the appropriate sterilization parameters for sterile filtration, steam sterilization, or dry heat sterilization. When bioburden testing is used in routine production, it would be considered a GMP test and would be fully validated and included in regulatory filings.

For aseptically filled injectable products, emphasis would be given to the numbers and size of the microorganisms in a presterile bulk solution and the size retention, bulk solution volume, and filtration area subject to sterile filtration. With moist or dry heat sterilization, the numbers of spores and their relative resistance, that is, \( D \)-value, to the sterilization process would be considered.

With presterile bulk solutions, the bioburden requirements would be more conditional depending on the bulk volume, nominal pore size, and the filter size than the nature of the product. The rating of a sterilizing filter is the retention of 10\(^7\) colony-forming units (CFU) of the challenge organism \( Brevundimonas diminuta \) per square centimeter of filter surface. It should be noted that the current EU guidelines for presterile bulk solutions are 10 cfu/100 mL, and tandem sterilizing filters are typically employed (CPMP/QWP/486/95, 1996).

### SORT OF RAPID MICROBIOLOGICAL METHODS

An RMM is an alternate microbiological test that is completed in shorter time than the classical tests that depend on incubation for microbial growth to detect microorganisms as either colonies on a plate or turbidity in a broth. It may involve reducing the incubation time for the plate count by at least half, processing a sample to obtain a result in two to three hours, or a direct analytical method. The latter two approaches are typically not growth-based, and hence move toward real-time analysis.

As pharmaceutical microbiologists, our primary objectives are to determine which microorganisms, if any, are in the pharmaceutical ingredients, intermediates, plant environment, or drug products and, if present, how many microorganisms and what microorganisms they are and their potential impact, to help the quality unit make decisions to proceed with the manufacturing and release of the product to the market. The test methods are classified as detection, screening, enumeration, and identification (Cundell, 2004). Examples from the compendial microbial tests are sterility testing (detection/qualitative), absence of specified microorganisms (screening/qualitative), and microbial count (enumeration/quantitative). In addition, there is the noncompendial microbial identification (identification/qualitative).

The classification systems for rapid methods proposed in the PDA Technical Report No. 33 are based on what the technology involves, for example, the growth of microorganisms, viability of microorganisms, presence/absence of cellular components or artifacts, nucleic acid methods, traditional methods combined with computer-aided imaging, and combination methods (Parenteral Drug Association (PDA), 2000; Moldenhauer, 2005). Similar, but slightly different classifications may be found in compendial sections discussing the validation of alternative microbiological test methods (USP 1223; European Pharmacopoeia 5.1.6, 2006).

### Growth-Based Technologies

These methods are based on measurement of biochemical or physiological parameters other than turbidity or colony formation, used in classical methods that reflect the growth of the microorganisms. Examples include ATP bioluminescence, colorimetric detection of carbon dioxide production and measurement of change in head-space pressure, impedance, advanced imaging, and biochemical assays.
Viability-Based Technologies
These types of technologies do not require growth of microorganisms for detection. Differing methods, including vital staining and fluorogenic substrates, are used to determine if the cell is viable or nonviable, and, if viable cells are detected, they can be enumerated. Examples of this technology include solid-phase cytometry and flow fluorescence cytometry.

Cellular Component or Artifact-Based Technologies
These technologies look for a specific cellular component or artifact within the cell for detection and/or microbial identification. Examples include fatty acid profiles, matrix-assisted desorption ionized-time of flight (MALDI-TOF) mass spectrometry, enzyme-linked immunosorbent assay (ELISA), fluorescent probe detection and bacterial endotoxin LAL test.

Nucleic Acid-Based Technologies
These technologies use nucleic acid methods as the basis of operation for detection, enumeration, and/or identification. Examples include DNA probes, ribotyping polymerase chain reaction (PCR), and ribosomal DNA-based sequencing.

A SURVEY OF RAPID MICROBIOLOGICAL METHODS
In most cases, RMMs may be divided according to their principle of detection. In this survey of RMMs, a ranking is made on the basis of successful implementation in the pharmaceutical industry (Tables 1 and 2). These are provided as useful information to the reader and not intended to be an endorsement from the author. Other systems may be available or become available that are not included in the table.

It can be concluded that some of the most successful RMMs are the ChemScan, AkuScreen, and BacT/ ALERT systems. More companies use these RMMs for in-process controls than product release. The latter is often product dictated. The rate of success of the implementation is determined by the ability to focus and reserve manpower in the qualification and validation work.

**ON REAL-TIME MICROBIOLOGICAL METHODS**
In general, decision makers (i.e., physicians, production managers, and quality units) claim that the microbiological testing laboratories in the hospitals, food production sector, and pharmaceutical industry are the rate-limiting steps for patient treatment and product release. As microbiologists, we recognized the truth in their criticism that microbial tests are imprecise with long incubation times. In Table 3, typical incubation times are shown for a range of microbial tests.

Microbiology laboratories count the time in days or even weeks to obtain a result. Furthermore, the results may need to be interpreted, reviewed, and approved before they can be reported. That is not all. The time to ship the samples to the laboratory must be considered. It is a simple addition calculation: Time to report = Time to ship the sample to the laboratory + administrative time + analysis time + incubation time + verification time + approval time + time to report the result. Product

| TABLE 1. Some Representative RMM Frequently Implemented in the Pharmaceutical Industry |
|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| System                                      | Technology                                      | Major application                              |
| ChemScan RDI                                | Solid-phase LASER fluorescence scanning microscopy | AVC                                           |
| MicroPRO (RBD 3000)                         | Fluorescence flow cytometry                     | AVC, PA                                       |
| RapiScreen/AkuScreen                        | ATP bioluminescence                             | P/A                                           |
| BacT/ALERT                                  | CO₂ colorimetric detection                      | P/A                                           |
| Pallcheck                                   | Membrane filtration ATP bioluminescence         | P/A                                           |
| BACTEC 9000                                 | CO₂ detection fluorescence                     | P/A                                           |
| Endosafe PTS                                | Handheld chromogenic                            | Bacterial endotoxin assay                     |
|                                              | LAL endotoxin assay                             |                                               |

Abbreviations: AVC, aerobic viable count; P/A, presence/absence; LAL, *Limulus* amebocyte lysate.
Other important differences that we recognize are among the in-process RMM testing for production process control, RMM testing for troubleshooting, and RMM testing for product release. All three may have different goals. Some definitions are in order before discussing the goals. The normal way we perform microbial analysis when the sample is taken to the microbiology laboratory is called off-line testing, if an analysis takes place near the production line but the sample is taken out of the production process, it is called at-line testing, and the last type is in-line testing, where there is a continuous analysis ongoing in the production process.

Conventional microbial testing, in most cases, is off-line testing with a few cases of at-line testing (depending on the manufacturing infrastructure). If we examine RMMs, they also belong to these two categories with some exceptions that have the potential to be used in-line (refer to Table 2 for an overview of different RMMs).
What determines whether an RMM can be used off-line, at-line, or in-line? In most cases, it is the underlying principle of the technique. For this discussion, RMMs can be subdivided into different categories on the basis of their detection principle: (1) detection of early growth, (2) viability-based testing, and (3) detection of microbial cell components. RMMs based on the early detection of growth principle are the slowest; the other two will be faster depending on the kind of application. As an example: detection of CO$_2$ production is a growth-based detection principle. In fact, there are some techniques available that are potential in-line detection systems based on viabile cell detection. In the last category, detection of cell components has many applications: detection of DNA, fatty acids, ATP, etc. In most cases, it requires a sample preparation that automatically converts it to an off-line application. There are several examples of at-line detection of bacterial endotoxins that may be used in pharmaceutical manufacturing.

Can we conclude that the only real-time RMM is a system that is based on viability cell detection in an in-line PAT application? (time to report = real-time result) In principle, the answer is yes. However, in most cases, it is not possible to use the viability cell detection principle in-line. The best and most practical possible option for the production departments and microbiology laboratories that serve them would be at-line testing with a viability-based cell detection principle (time to report < 30 minutes). However, because the viability-based cell detection systems are technically limited in their lack of sensitivity (limit of detection/quantification) and specificity (differentiating between cells and particulates), we end up with an at-line testing option of detection of early growth or cell components to eliminate ambiguity (time to report 24-48 hours).

It must be emphasized that with RMMs, the objective of the testing determines what kind of system is needed. For RMM testing for product release for the market, an off-line testing system is the right choice because there is no need for testing at the production floor. RMM testing for troubleshooting, in contrast that for product release, can be both at-line testing and off-line testing. RMM testing for in-process testing would be preferably done at line. With the latter, the difficulty and workability of a test method determines the at-line or off-line application of a test.

THE APPLICATION OF RMM TO ASEPTIC PROCESSING

Aseptic processing may be divided into: (1) aseptic bulk processing most often employed with biologics and (2) aseptic filling and lyophilization with both biologics and small molecules. On the basis of a risk assessment, critical control points can be established and, if necessary, monitored to minimize the risk of microbial contamination and loss of environmental control (Cundell, 2005). This monitoring would be more effective if conducted in real time to provide the opportunity to take corrective action to reduce the possibility of contamination.

The following microbial tests may be used during in-process monitoring:

1. Microbial limits and bacterial endotoxin testing of incoming pharmaceutical ingredients and packaging components
2. Microbial counts and bacterial endotoxin testing of water for pharmaceutical use, buffers, and other intermediates
3. Presterile filtration bioburden monitoring
4. Biological indicator monitoring
5. Sterility testing of sterile bulk drug substances
6. Microbial monitoring of air, surfaces, and personnel in clean rooms

Bacterial Endotoxin Testing

As pointed out earlier, with endotoxin monitoring, two major innovations are notable. They are handheld bacterial endotoxin monitoring units (Endosafe) that are used by manufacturing personnel to test the water for injection points of use immediately prior to delivering ingredient water and in at-line monitoring systems (PyroSense) that continuously monitor endotoxin levels in a water-for-injection loop at preset time intervals. These instruments can mitigate the risk of using endotoxin-contaminated water.

Water Testing

Alert and action levels are set and microbial counts are used to monitor pharmaceutical water systems to identify possible out-of-trend conditions that require corrective action. The monitoring can identify potential point-of-use, loop, or entire water system problems. As the European requirements specify the use of membrane filtration with R2A agar incubated at 30 to 35°C for at least five days (the author recommends lower temperatures with a longer incubation period, i.e., 20-25°C for 7-10 days), microbial excursions are identified long after the ingredient water has been used. RMMs that have been used for monitoring water systems include the Milliflex Rapid System based on membrane
filtration, ATP bioluminescence and advanced imaging, the Scan RDI system based on membrane filtration, a fluorogenic substrate and solid phase LASER scanning microscopy, and the Micro Pro System based on vital stain flow cytometry. These systems may be used to obtain microbial counts within the order of 18 hours, 3 hours, and 30 minutes, respectively. Of these technologies, only the flow cytometry system meets the definition of real-time, at-line testing suitable for a PAT application, although the method may lack sensitivity (level of quantification on the order of 100 bacteria per mL) for many applications that depend on enumeration and not just screening for gross contamination.

Bioburden and Sterility Testing

For aseptically filled injectable products, emphasis would be given to the numbers and size of the microorganisms in the presterile bulk solution, the volume of bulk solution to be filtered, and the size retention and filtration area of the sterilizing filter. The rating of a sterilizing filter is based on the retention of 10^7 colony-forming units (CFU) of the challenge organism B. diminuta per square centimeter of filter surface. As noted earlier, the current EU guidelines for presterile bulk solutions are 10 CFU/100 mL, and tandem sterilizing filters are typically employed in Europe. With tandem sterilizing filters, monitoring the bioburden of the bulk solution challenging the second filter may be eliminated. To demonstrate that the bulk solution meets this requirement, a 100-mL sample would be tested using a membrane filtration method. Given the stringent requirements, RMMs must have a limit of detection and quantification commensurate with the 10 CFU/100 mL limit as well as a rapid turnaround time. This severely limits the options available for bioburden monitoring.

A possible option is to use a RMM as a presence/absence test for water for injection, low pyrogen purified water, and in-process material to screen out samples that contain no microorganisms where processing would continue and concentrate on additional enumeration of those samples that contain microorganisms.

Sterility testing of sterile bulk drug substances and sterile bulk solution prior to aseptic filling is typically conducted using a 10-mL sample inoculated into broth and incubated for at least 14 days. With sterile drug substances that are being stored for future use, there is no time constraint for sterility testing unless there is a need to reprocess the drug substance, to prevent product loss, if it is found not to be sterile. Sterile bulk sterility tests are legal requirements for biologics marketed in the United States, as described in 21 CFR 610.13.

The MicroCompass™ Detection system based on the detection of universal sequences of RNA using a one-step real-time reverse transcriptase PCR assay and MGB® Eclipse probe technology is a promising new option. Universal sequences detected are based on ribosomal 16S rRNA (bacteria) and 18S rRNA (yeast and molds). The sensitivity is 50 fg of RNA or as little as 100 CFU. This test system has a detection limit that shows sensitivity to the edge of the bioburden limit.

Environmental Monitoring

Microbial monitoring of air, surfaces, and personnel in clean rooms is conducted during each manufacturing shift. The results are delayed for five to seven days due to the incubation of the microbiological culture media. As environmental monitoring is by far the largest type of microbial testing in an aseptic filling facility, the automation of the sampling, incubation, and reading of plates would increase the efficiency and timeliness of the monitoring. A test system that can achieve this goal is the Growth Direct System based on the early detection of microcolonies on plates using advanced imaging.

A test system that will achieve real-time environmental monitoring is the Biovigilant Air Monitoring System that is capable of counting both viable and nonviable particles in a clean room setting. This may be used as a PAT application detecting with high-efficiency particular air (HEPA) filter failures, isolator system leaks, human interventions generating airborne microorganisms, and the ingress of microorganisms from supporting areas. Such real-time detection would enable immediate corrective action such as line clearance, changes in clean room behavior, and even aborting aseptic filling operations.

**THE APPLICATION OF RMM TO BIOPHARMACEUTICAL UPSTREAM AND DOWNSTREAM PROCESSING**

In bioprocessing, microbiological control plays an important role. The definition of bioprocessing is important. Bioprocessing is the manufacture of therapeutic proteins using mammalian, bacterial, yeast, or other living (plants, insects) cells. This process can be divided into two parts: (1) upstream processing, in which the cell culture step takes place, and (2) downstream processing, where the protein is recovered and purified using a range of biochemical purification techniques, especially large-scale column chromatography.

The scale of the bioprocessing has increased in the last 10 years. It started with small-scale cultures of 10 L but increased to larger volumes of the order of 15,000 L. The challenge to prevent contamination of those giant fermentors is huge. Financial risks are high ($65 /L medium, which means that the costs of only one contaminated fermentor can be of the order of $970,000).

Looking at the downstream processing, we see the
same kind of evolution in scale. It started with small columns but currently involve the use of large columns, and the associated resins are used that are expensive to maintain and difficult to replace once contaminated. The golden rules in the bioprocessing industry encompass the following:
1. Prevent contamination from input materials and equipment.
2. Detect contamination as quickly as possible.
3. Monitor your process with critical control points.
4. Take corrective action as soon as possible to isolate the incidence of contamination and find the root cause.

Sterile media and equipment is achieved using validated sterilization processes and released by the use of a validated rapid microbial method. To be useful, RMMs must generate real-time results within the processing area and not a microbiology laboratory.

If the decision is taken to implement RMM in bioprocessing operations, a series of steps has to be taken to prove the PAT concept. Most important is the first step: the selection of "the most valuable sample," or in risk analysis terminology, the critical control point. These are the key samples that mark a critical step in the process. For example, before the inocula are transferred from a smaller to the next larger fermentor, it is wise to take a sample before the processing reaches the scale of 15,000 L. It goes without saying that all the input materials (media, buffers, cells, compressed air, etc.) are critical samples. If contamination occurs, RMMs are very useful to troubleshoot the process. The first 24 hours after contamination occurs is vital. The longer it takes to collect and analyze data, the more difficult it will be actually to find the root cause of the contamination and take corrective action. Another important tool for root cause analysis is a rapid identification technology. The identity of a microbial contaminant can help to find the root cause. Rapid identification, that is, within one day, can be very useful. Automatically, a genotyping-based technology will be the method of choice, for example, 16s rRNA sequencing, due to its rapidity and accuracy.

THE ROLE OF RMM IN ENVIRONMENTAL MONITORING AND CONTROL

What is the role of environmental microbiological monitoring? In general, monitoring is performed to get insight into the microbiological quality of the manufacturing environment. Depending on the classification of the production environment, critical locations are selected and are sampled by contact plates, settle plates, or active air monitoring. Monitoring can be divided into monitoring of surfaces, air, and personnel. The specifications of the monitored places depend also on the classification of the area and the criticality of the operation. Strict limits are used in a grade A area (ISO 5) (<1 cfu/settle plate), whereas grade B (ISO 6-8) or lower classified areas have less stringent limits. As incubation times are long for monitoring media (3-5 days), the results represent the past history of that sampled area and not the current status. That is widely recognized in the industry; hence, we follow the trend of the microbiological cleanliness with respect to sampling times. As soon as an adverse trend is detected in the microbiological quality of a sampling location, corrective action is taken such as additional disinfection, retraining of the personnel, or screening for changes in the environment. Immediate action to remedy an out-of-limit result in monitoring in general is difficult because of the time lag in the actual monitoring action and the time the result is known.

The results that are obtained with the current monitoring techniques give, as expected, a relative value. Monitoring efficiency depends on the type of surface, the contact time, the type of media, and incubation time. This also adds up to the relative value of environmental monitoring, and stresses the importance of performing trend surveys to assure control of the microbiological quality of the environment.

What is then the role of RMMs in environmental monitoring? The conventional methods give a good insight into the microbiological quality of the environment; however, they have the disadvantage that errors, for example, a wrong disinfection procedure, are detected at a later point or not even detected at all. That points to the possible benefit of RMM in environmental monitoring. A timely corrective action can be performed and the risk of production in a dirty environment is diminished. RMMs contribute to the validated state of the production process. The link to the actual product batch that is being produced is difficult to make with environmental monitoring. If production takes place in a microbiologically dirty environment, the chance of getting a contaminated product is higher. If RMM is used, it may be easier to link the actual microbiological measurement to the microbiological quality of the product. Parametric release could be easier using these RMM technologies.

At this moment, there is no definitive RMM for environmental monitoring available which gives results the same day. Direct cell detection by ChemScan/ScanRDI technology has been tested by some companies for air monitoring but is not a widely used application because of the low throughput and cost in testing with this technology. ATP measurement could be the method of choice, as instrumentation is available that can process many samples and the technology has been successfully used for hygiene monitoring in the food industry.
However, it is not sensitive enough to measure low microbial counts on the very clean surfaces that are common in pharmaceutical production. The ultimate RMM for environmental monitoring should give results within 30 minutes, be quantitative and very easy to operate in a clean room environment.

**INDUSTRY, REGULATORY, AND COMPENDIAL GUIDELINES FOR RMM**

After the PDA Technical Report No. 33 was published (PDA, 2000), a number of regulatory and compendial documents have been issued that were strongly influenced by the technical report to address the selection, purchase, implementation, and regulatory submission of alternate microbiological methods including RMMs. They include the following.

1) PDA Technical Report No. 33

The PDA was the first organization to develop guidance for the evaluation, implementation, and validation of RMMs. PDA Technical Report No. 33 was developed by a committee of individuals from the industry, regulatory agencies, compendial groups, and instrument vendors and chaired by one of the authors of this chapter. This guidance provided definitions in microbiological terms for validation criteria similar to the information in USP <1225> for chemistry methods.

2) USP Informational Chapter <1223> on Validation of Alternative Microbiological Methods

The USP Informational Chapter <1223> defined the validation criteria to be used for RMMs, along with definitions of these criteria in terms of microbiology, in contrast to chemistry as found in USP <1225> (USP 1223). The proposal also identifies how to determine which criteria are applicable to different technologies, on the basis of the type of testing being performed.

3) GMPs for the 21st Century

The FDA initiated a program to modernize requirements for pharmaceutical manufacturing and quality. This modernization included encouraging the early adoption of new technologies, facilitation of industry application of modern quality management technologies, encouraging the implementation of risk-based approaches in critical areas, ensuring that policies for review of submissions, compliance, and facility inspections are based on state-of-the-art technologies, and enhancing the consistency and coordination of FDA regulatory programs. This resulted in an initiative titled “Pharmaceutical cGMPs for the 21st Century-A Risk-Based Approach” in 2004 (FDA 2004b).

4) FDA Guidance on Aseptic Processing 2004

FDA published an updated guidance document on aseptic processing of pharmaceutical products. It includes a provision for the use of alternative microbiological test methods (FDA, 2004c). This guideline was titled "Guidance for Industry Sterile Drug Products Produced by Aseptic Processing-Current Good Manufacturing Practice".

5) Ph. Eur. Chapter on RMM

The Ph. Eur. published 5.1.6. “Alternative Methods for Control of Microbiological Quality” (European Pharmacopoeia 5.1.6., 2006). This document provided an overview of some RMMs available and potentially applicable to pharmaceutical processes, and how they may be used for microbiological control of products and processes. It also provides guidance on how to choose and validate an appropriate method using the ATP bioluminescence technology as an example.

**THE SELECTION, DEVELOPMENT, VALIDATION, AND IMPLEMENTATION OF AN RMM FOR PROCESS ANALYTICAL TECHNOLOGY APPLICATIONS**

The implementation of an RMM in the production area is a considerable challenge, although it is becoming easier compared with the situation five years ago. This process can be divided in different steps to be taken, which are important to follow in order to ensure a successful implementation. The goal of implementation of each rapid method can be different (like earlier mentioned). A reduction of cycle time is a common goal. In this case, the testing will be conducted on the end product of the production process. Another goal is to mitigate risks of microbial contamination in the production process (preventative) and avoid troubleshooting failures in determining the root cause (reactive). In this case, the RMM is assurance against microbial contamination and will safeguard the production process. The following steps should be taken:

1) Discuss in detail with the manufacturing the details of the production process and select the most valuable sample or critical control points.

2) Select the most suitable detection method (growth based, direct cell detection, or detection of cell components) that is compatible with the nature of the sample, the expected contamination, and the sensitivity to be achieved.

3) Select the instrumentation that best fits the sample and the technology.

4) Select an equipment supplier.

5) Perform pilot or proof-of-concept testing to prove that the instrumentation fits the specific application. Perform method suitability testing for a range of test materials.

6) Purchase the instrumentation and perform the equipment validation, that is, Installation Qualification (IQ), Operational Qualification (OQ), and Performance
Qualification (PQ) using vendor supplied documents whenever possible.
7. Perform method suitability testing at least on three independent batches.
8. Assemble all the GMP documentation (Standard Operating Procedures, calibration programs, regulatory submissions, and change controls).
9. Implement in routine testing.

REGULATORY APPROVAL OF RMM

With the FDA, three avenues are possible for the approval of RMMs. A New Drug Application (NDA) submission for an RMM may be used with a new product and an NDA supplement for an existing product, filing a comparability protocol, or using the PAT initiative pathway. The FDA prefers the comparability protocol approach (FDA, 2003) as it accommodates the fact that the FDA to approvals are typically drug product specific, and a comparability protocol gives the FDA the opportunity to review the current method validation plan prior to its execution for a range of drug products. In general, it is advisable to discuss the application and validation strategy with the regulatory agency in advance.

The most important RMM validation issue is equivalence to the current method. Other standard validation issues include accuracy, sensitivity, precision, and linearity of response. Microbiologists should use supplier-generated validation protocols whenever possible. IQ is best timed with the delivery of the equipment to your laboratory. OQ will demonstrate the functionality of the equipment while PQ will be directly related to your application and products. Remember it is acceptable to include supplier-generated reports and publications from peer-reviewed journals within your validation report so that you may avoid repeating the generation of pre-existing data. Validation protocols and reports must include the validation rationale, acceptance criteria, and deviations from protocol or acceptance criteria, and the documents must be reviewed and approved by the quality unit.

THE FUTURE OF RMM IN PARENTERAL MEDICATION MANUFACTURING

What is the future of RMMs in parenteral drug manufacturing? The major trends are (1) the move away from traditional growth-based methods to RMMs on the basis of vital cell staining, ATP, or nucleic acid concentration, (2) the move from the microbiology laboratory to the production floor as the site of the microbial testing, and (3) the use of RMM for PAT applications by the real-time testing of in-process samples.

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