

Rapid Assay of Bioburden, Endotoxin and Other Contamination

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Abstract

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 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 (RMM) 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 proved 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 count detected by incubation method and rapid assay has great divergence.

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 implementation of alternative microbial methods.

Keywords: 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 [1]. 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 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; the selection, development, validation, and implementation of RMM for PAT applications; industry, regulatory, and compendia guidelines for RMM; 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 quality and efficiency in the pharmaceutical industry. Those

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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 was traditionally been conducted by inoculating a microbiological broth with time 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 requirement from the different pharmacopoeias were included in the compendial tests and these will be 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, lipopolysaccharide, present in the by 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 amoebocyte lysate* (LAL) endotoxin test in the mid-1970s making the test suitable for both in-process and finished product testing. This test is largely responsible for the elimination of pyrogens from parenteral drug products. As different sources of endotoxin have differing potency, the standard was assigned potency in endotoxin units (EU).

The compendial bacterial endotoxins 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-fold dilution where clot formation occurs. Despite this limitation, in the event of a dispute as to the endotoxin content of a product, the referee test is the

longer established gel-clot method. In general, a kinetic turbidimetric or chromogenic method would be preferred to the gel-clot method to fully quantify the bacterial endotoxin and remove the subjectivity of the gel-clot method using dilution one-half, one-quarter, or one-tenth of the maximum valid dilution. As the incubation period is one hour or possibly less, endotoxin screening has a high potential as a PAT application.

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 solutions, 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 undertaken as part of a risk assessment during formulation and manufacturing process development. For example, as part of 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 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 [2].

Sort of Rapid Microbiological Methods

A 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 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, hence move toward real-time analysis.

As pharmaceutical microbiologists, the primary objectives are to determine which microorganisms, if any, are in the pharmaceutical ingredients, intermediates, plant environment, or drug products; if present, how many microorganisms and what microorganisms they are and their potential impact, to help the quality unit make decisions to proceed with manufacturing and release product to the market. The test methods are classified as detection, screening, enumeration, and identification [3]. 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 how the technology works, for example, 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 [4,5]. Similar, but slightly different, classifications may be found in compendial sections discussing the validation of alternative microbiological test methods [6,7].

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 into classes on the basis of 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 BacTI ALERT systems. More companies use these RMMs for in-process controls than product release. The latter is often product dictated. Rate of success of implementation is determined by the ability to focus and reserve manpower on the qualification and validation work.

On Real-Time Microbiological Method

In general, decision makers (i.e., physicians, production managers,

System	Technology	Major application
ChemScan RDI	Solid-phase LASER	AVC
fluorescence scanning microscopy		
MicroPRO (RBD 3000)	Fluorescence flow cytometry	AVC, P/A
RapiScreen/AkuScreen	ATP bioluminescence	P/A
BacT/ALERT	CO ₂ colorimetric detection	P/A
Pallchek	Membrane filtration ATP bioluminescence	P/A
BACTEC 9000	CO ₂ detection fluorescence	P/A
Endosafe PTS	Handheld chromogenic	Surface monitoring
LAL endotoxin assay	Bacterial endotoxin assay	

Abbreviations: AVC, aerobic viable count; P/A, presence/absence; LAL, Limulus amebocyte lysate.

Table 1: Some Representative RMM Frequently Implemented in the Pharmaceutical Industry.

System	Technology	Major application
Q-PCR: Micro Compass	RT-PCR	AVC
Bac Trac 4300	Impedance	AVC, P/A
Soleris Pathogen Detection Systems	Optical biochemical	AVC
RABIT	Impedance	AVC
Pyro Sense	Chromogenic LAL, recombinant Factor C based endotoxin	On-line endotoxin
Biovigilant Air Monitoring System	Direct cell detection	On-line air monitoring
Growth Direct imaging	Autofluorescence Advanced	AVC
Kikkoman ATP Swabsfor Hygiene Testing	ATP detection	Surface monitoring

Abbreviations: AVC, aerobic viable count; P/A, presence/absence.

Table 2: Some Representative RMM with the Potential to Be Implemented in the Pharmaceutical Industry.

Test	Incubation time
Total aerobic microbial count	3-5 days
Total yeast and mold count	5-7 days
Sterility tests	14-21 days
Absence of specified microorganisms tests	18-72 hrs
Limulus amebocyte lysate endotoxin tests	1 hr
Microbial identification, phenotypic	3-5 days
Microbial identification, genotypic	1 day
Preservative efficacy tests	
Mycoplasma test	28 days

Table 3: The Incubation Requirement for Microbial Tests Used in Drug Manufacturing.

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 criticisms 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. And 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+verify time+approval time+time to report the result. Product release cycle times are protracted and are the sum of all these sequential activities. That means seven [7] items to work on to speed up the overall

testing process. With RMM, in most cases, only the analysis time and incubation time is considered. It is important not to forget the other time-consuming factors in the analytical process when considering RMM implementation.

Other important differences that we recognize is that among 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 one 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 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 now whether a 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. Some examples: Detection of CO₂ production is a growth-dependent technique that may be used for sterility testing. This application is unlikely to be an in-line application because of the aseptic handling that is inherent to the sterility test. In best case, it could be an at-line application. Detection via flow cytometry has a viability based detection principle. Although it is not on the market, we can imagine that an in-line application could be possible to detect and count microorganisms via a laser detection principle. In fact, there are some techniques available that are potential in-line detection systems based on viability cell detection. The last category: Detection of cell component 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 endotoxin that may be used in pharmaceutical manufacturing.

Is the conclusion 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. What is the best possible option for the production departments and microbiology laboratories that serve them? The most practical option 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 have the technical limitations of a 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/cell component 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 to 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 [8]. 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 water for injection points of use immediately prior to delivering ingredient water and at-line monitoring systems (PyroSense) that continuously monitor endotoxin levels in a water-for-injection loop at preset time intervals. These instruments can mitigate risk of using endotoxin-contaminated water.

Water testing

Microbial counts are used to monitor pharmaceutical water systems for alert and action levels 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 temperature with longer incubation period, i.e., 20-25°C for 7-10 days), 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 be too insensitive (level of quantification on the order of 100 bacterial cells 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 a 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 the retention of 10⁷ 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 requirement, 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 sample 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 to be not sterile. Sterile bulk sterility tests are legal requirements for biologics marketed in the United States, using the tests according to 21 CFR 610.13.

The MicroCompass® Detection system based on detection of universal sequences of RNA using a one-step real-time reverse transcriptase PCR assay and MGBR Eclipse probe technology is a promising new technology. 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 technology has a detection limit that has sensitivity on the edge of 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 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 technology that will achieve this goal is the Growth Direct System based on the early detection of microcolonies on plates using advanced imaging.

A technology 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 high-efficiency particulate air (HEPA) filter failures, isolator system leaks, human interventions generating airborne microorganisms, and the ingress of microorganisms from supporting areas that 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 the bioprocessing, microbiological control plays an important role. The definition of the 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 bioprocess has increased in the last 10 years. It started with small-scale culture <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 only the costs of one contaminated fermentor can be of the order of 970K \$).

Looking at the downstream processing, we see the same kind of evolution in scale. It started with small columns and currently large columns, and their associated resins are used that are expensive to maintain and difficult to replace once contaminated.

The golden rule in bioprocess industry is the following:

1. Prevent contamination from input materials and equipment.
2. Detect a contamination as fast as possible.
3. Monitor your process on critical control points.
4. Take corrective action as soon as possible to isolate the incidence and find the root cause analysis.

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 have 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, RMMs, compressed air, etc.) are critical samples. If a contamination occurs, RMMs are very useful instruments to troubleshoot the process. The first 24 hours after a 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. An important tool for root cause analysis is also 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 an out of limit 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 manufacturing errors, for example, a wrong disinfection procedure, are detected at a later point or not even detected at all. That could be the 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 batch of product 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 was tested by some companies for air monitoring but is not a widespread 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, the sensitivity is insufficient 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 and is 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 [4], 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 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 Information 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 early adoption of new technologies, facilitation of industry application of modern quality management technologies, encouraging implementation of risk-based approaches in critical areas, ensuring that policies for review of a submission, compliance, and facility inspection 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 [9].

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 [10]. 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 [7]. 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 situations five years ago. This process can be divided in different steps to be taken, which are important to follow to assure 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 risk mitigation for microbial contamination in the production process (preventative) and troubleshooting failures to determine 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 fits the best for 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 document 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 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 executing it 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. Microbiologist 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 publication from peer-reviewed journals within your validation report so you may avoid repeating the generation of preexisting 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 to PAT applications by the real-time testing in-process samples.

References

1. Guidance for Industry-PAT A Framework for Innovative Pharmaceutical Development, TI Manufacture and Quality Assurance (2004). Food and Drug Administration.
2. Note for Guidance on Manufacture of the Finished Dosage Form (1996). Committee for proprietary Medicinal Products (CPMP).
3. Cundell AM (2004) Microbial testing in support of aseptic processing. *J Pharm sci Technol* 56-66.
4. <http://www.rapidmicrobio.com/Portals/54227/docs/TR33.pdf>
5. Moldenhauer J (2005) Rapid microbiological methods and the PAT initiative. *Biopharm Int* 18: 31-46.
6. Validation of Alternative Microbiological Methods.
7. Alternative Methods for Control of Microbiological Quality (2006). European Directorate for the Quality of Medicines, Strasbourg, France.
8. Cundell, AM (2005) Risk-Based Approach to Pharmaceutical Microbiology. In *Encyclopedia of Rapid Microbiological Methods*. Miller MJ, ed, Bethesda, MD: Davis Horwood/PDA.
9. Pharmaceutical cGMPs for the 21st Century-A Risk-Based Approach, Final Report (2004). Department of Health and Human Services, U.S. Food and Drug Administration.
10. Sterile Drug Products Produced by Aseptic Processing-Current Good Manufacturing Practice (2004). Guidance for Industry, Department of Health and Human Services, U.S. Food and Drug Administration.