Rapid microbiological methods 17

17.1 Introduction

Rapid microbiological method (RMM) technologies aim to provide more sensitive, accurate, precise, and reproducible test results when compared with conventional, growth-based methods. RMMs may be interpreted to include any microbiological technique or process that increases the speed or efficiency of isolating, culturing, or identifying microorganisms when compared with conventional methods.

Although the use of the word "rapid" is often used to describe the range of techniques employed, some of the methods included here do not give a more rapid result but instead a more accurate, precise, or detailed result, so providing more information on which to base a decision. Alternative methods are sometimes referred to in relation to rapid methods, although alternative methods need not necessarily be "rapid." Where a compendia test is described (such as the sterility test), it is assumed to be the standard method, and from this, an alternative method is simply one that is different from the method described in the pharmacopoeia.

Rapid methods normally involve some form of automation, and the methods often capture data electronically. With several different technologies available on the marketplace, the microbiologist has a difficult, and sometimes expensive, choice to make in selecting the optimal method. When considering rapid methods, the new method must offer a higher level of quality assurance. There needs to be a clear and demonstrable benefit in adopting the alternative method. Several advantages are discussed in this chapter.

This chapter, while addressing some of the emerging technologies, is not so much about the different RMMs that are available; it is more concerned with the considerations that are needed for their selection. As such, the chapter provides some advice for the microbiologist to consider when drawing up a rationale for the selection of a rapid or alternative microbiological method. The chapter does describe different technologies, but it does not describe different commercial companies (to do so could date the chapter and could risk giving preference to a system that is not necessarily the most robust). The reader with an interest in a given technology and application should consult contemporaneous literature.

17.2 Changing world of microbiology

Conventional microbiological methods, including those long-established and described in the European (Ph. Eur.), Japanese, and US Pharmacopeia (USP), have served microbiologists well over the past century and have helped to ensure the production of microbiologically safe products. For example, a wide range of microbiological methods has been successfully verified using plate count methods to enumerate and identify

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microorganisms (within an accepted margin of error [1]). However, conventional methods have limitations. These limitations include the time taken to produce a result and the inability of many methods to recover all of the microorganisms that might be present in a sample.

Considering these issues further, the time taken to produce a result relates to the incubation period required for conventional methods, which rely on agar as a growth medium, or for microorganisms to grow in broth culture. Such methods are relatively slow, and results are only available after an incubation period (somewhere between 2 and 10 days, depending on the application) [2].

A further limitation is culturability and the issue of viable but nonculturable (VBNC) microorganisms. Many bacteria, although maintaining metabolic activity, are nonculturable due to their physiology, fastidiousness, or mechanisms for adaptation to the environment. Some research suggests, for example, that less than 10% of bacteria found in cleanrooms are culturable [3]. Thus, it stands that some RMMs, especially those that do not rely on growth, may provide a higher recovery count as compared with traditional methods. Some rapid methods produce results where the number or types of microorganisms can be measured. With rapid methods that do not directly "grow" microorganisms, such as those that detect metabolic activity, it is possible to correlate the new measurements, such as a fluorescing unit, with the old measurement (i.e., the colony-forming unit; CFU) and establish new acceptance levels.

These concerns with limitations of conventional methods, as well as the possibilities afforded by technological advances, led to an emerging new generation of rapid and alternative microbiological methods. RMMs and alternative microbiological methods include any microbiological technique or process that increases the speed or efficiency of isolating, culturing, or identifying microorganisms when compared with conventional methods [4].

As to what rapid methods are, according to the US Food and Drug Administration (FDA) [5]:

> *RMMs are based on technologies which can be growth-based, viability-based, or surrogate-based cellular markers for a microorganism (i.e., nucleic acid-based, fatty acid-based). RMMs are frequently automated, and many have been utilized in clinical laboratories to detect viable microorganisms in patient specimens. These methods reportedly possess increased sensitivity in detecting changes in the sample matrix (e.g., by-products of microbial metabolism), under conditions that favour the growth of microorganisms.*

Although the use of the word "rapid" is often used to describe the range of techniques employed, some of the methods included within this collective do not give a more rapid result; they instead provide a more accurate, precise, or detailed result (and thus the term "alternative" is employed).

RMMs can be applied to a range of microbiological tests, including raw materials, water, intermediate products, final products, and environmental monitoring. There is a sufficient range of RMMs to provide an assessment of the microbiological quality throughout an entire production operation. RMMs may also be used by research and development. For example, in understanding formulations of products better in terms of whether microorganisms are likely to survive or be killed.

RMMs are essentially used as alternatives to four major types of conventional microbiological determinations [6–8]:

- qualitative tests for the presence or absence of microorganisms (e.g., enrichment turbidity measurements of growth). For example, to determine if *Escherichia coli* is in a sample of water;
- quantitative tests for enumeration of microorganisms (e.g., plate count methods to determine the bioburden of a sample);
- quantitative tests for potency or toxicity (e.g., what level of endotoxin is in the sample?);
- identification tests (e.g., biochemical and morphological characterization).

17.3 Advantages of rapid methods

Looking at some of the advantages afforded by rapid methods further, aside from the time-to-result, another important area is throughput. Most rapid systems allow for higher volumes than the traditional method. In environments with considerable volumes of raw ingredients, in-process batches and final products to test, a high throughput can confer an important advantage for maintaining manufacturing up-time and moving an inventory as quickly as possible.

Furthermore, RMMs can assist with:

- designing more robust processes that could reduce the opportunities for contamination (fitting in with some quality-by-design objectives);
- developing a more efficient corrective and preventive action process;
- confirming that the process is in a continuous state of microbiological control through "real-time" monitoring (that meets some process analytical test objectives);
- assisting with continuous process and product improvement.

Other advantages include labor efficiency and error reduction. Reducing errors is one of the greatest potential benefits of rapid enumeration. While some methods require extra human intervention and, thus, create greater potential for mistakes, others automate the most error-prone processes. Microbial counting, incubation changeovers, and data entry can all become far more reliable given the right equipment.

Arguably, RMMs enable a proactive approach to be taken to instances of microbial contamination, especially in relation to out-of-specification results. Here, RMMs enable quicker responses to out-of-trend situations through providing real-time or near real-time results. This allows corrective actions to be taken earlier.

Furthermore, when considering an RMM, the new method must offer a higher level of quality assurance. There needs to be a clear and demonstrable benefit in adopting the alternative method. Examples of this include:

- the ability to make critical business decisions more quickly;
- the prevention of recalls through greater method sensitivity to microorganisms;
- the detection of "objectionable" microorganisms;
- recovery of higher or more accurate microbial numbers;
- potential reduced stock holding through faster release times;
- improvement in manufacturing efficiency;
- a more proactive, rather than reactive, decision making.

It is because of these advantages that RMMs are areas of considerable investment by vendors and attract interest from microbiologists.

17.4 Regulatory acceptance

RMMs are accepted by the major global regulatory agencies. For example, in 2011, the FDA published their new strategic plan entitled *Advancing Regulatory Science at FDA* [9]. In Section 17.3, the FDA seeks to "support new approaches to improve product manufacturing and quality." With regard to control and reduction of microbial contamination in products, the FDA supports those who:

- develop sensitive, high-throughput methods for the detection, identification, and enumeration of microbial contaminants and validate their utility in assessing product sterility;
- develop and evaluate methods for microbial inactivation/removal from pharmaceutical products that are not amenable to conventional methods of sterilization;
- evaluate the impact of specific manufacturing processes on microbial contamination;
- develop reference materials for use by industry and academia to evaluate and validate novel methods for detecting microbial contamination.

17.5 Types of rapid microbiological methods

RMM or alternative methods can be categorized into multiple means. One way is based on the technology or application. Here, based on the Ph. Eur., the methods can be grouped into six categories.

17.5.1 Growth-based methods

Growth-based methods are those where a detectable signal is usually achieved following a period of subculture (e.g., electrochemical methods). These methods generally involve the measurement of biochemical or physiological parameters that reflect the growth of microorganisms. These methods aim to decrease the time at which one can detect actively growing microorganisms. The methods continue to use conventional liquid or agar media. In summary, they include:

- Impedance microbiology (measurable electrical threshold during microbial growth). With these methods, the relationship between capacitance at the electrode surface and conductance from ionic changes in the media from the by-products produced during bacterial growth allows for the calculation of impedance. With this, increases in capacitance and conductance result in decreased impedance, which is indicative of bacterial growth;
- The detection of carbon dioxide. Such methods deploy an internal colorimetric carbon dioxide sensor. The sensor can be placed inside a media bottle (separated from the media by a semipermeable membrane). As carbon dioxide is produced by microbial metabolism diffuses across the membrane, it dissolves in water in the sensor and generates hydrogen ions, which result in a color change detected by a colorimetric detector. Light emitted by

the detector reflects off the sensor onto a photometer. The resulting voltage signal is proportional to the intensity of the reflected light and to the concentration of carbon dioxide in the media bottle. As an alternative, headspace pressure monitors work in a similar way. Here, headspace pressure platforms detect growth as a result of consumption or production of gases in the headspace of sealed media bottles causing conformational changes in the geometry of the septum;

- The utilization of biochemical and carbohydrate substrates. In a similar way, detectors present in media can detect microbial reactions;
- The use of digital imaging and auto-fluorescence for the rapid detection and counting of microcolonies. This method overcomes the problem with culture methods where the visualization of colonies requires several days. The rapid method uses lasers to scan microorganisms growing on membrane filters with optical imaging using a digital camera. This technique allows detection and enumeration of microcolonies within a few days. An advantage of this technique is that the microorganisms remain viable for identification after colonies become visible;
- Fluorescent staining and enumeration of microcolonies by laser excitation. This method is similar to the one described above;
- Selective media for the rapid detection of specific microorganisms. This standard approach can be automated through the detection of specific nucleic acid sequences Such systems break down samples at the genetic level, using a polymerase chain reaction (PCR) to detect bacteria and other microbes.

17.5.2 Direct measurement

Direct measurement is where individual cells are differentiated and visualized (e.g., flow cytometry). These methods generally use viability stains and laser excitation for the detection and quantification of microorganisms without the need for cellular growth. These methods include:

- demonstration of direct labeling of individual cells with viability stains or fluorescent markers with no requirement for cellular growth;
- flow cytometry (individual particles are counted as they pass through a laser beam) and solid-phase cytometry (staining and laser excitation method). With these technologies, the ability to stain microorganisms with dyes such as propidium iodide, which is impermeable to cells with intact membranes, and thiozole orange, which is permeable to all cells, allows a differentiation of viable and nonviable bacterial cells in fluid media.

17.5.3 Cell component analysis

Cell component analysis is where the expression of specific cell components offers an indirect measure of microbial presence (e.g., genotypic methods). These methods generally involve the detection and analysis of specific portions of the microbial cell, including adenosine triphosphate (ATP), endotoxin, proteins, and surface macromolecules. The methods include:

- ATP bioluminescence (the generation of light by a biological process). With this method, the addition of a substrate to a membrane surface yields fluorescence following exposure to microbial ATP. This is because ATP is the main chemical energy source of all living cells.

Detection systems based on the bioluminescence exploit the chemical release of ATP from microorganisms. ATP reacts with luciferase and a photon counting imaging tube detects photons released by this reaction. A computer monitor then represents the photons detected. There are both qualitative and quantitative systems available;
- Endotoxin testing (*Limulus* amebocyte lysate; LAL). As Chapter 11 describes, automated LAL testing can provide results within minutes regarding the presence of bacterial endotoxin in a sample;
- Fatty acid analysis (methods that utilize fatty acid profiles to provide a fingerprint for microorganism identification). This method is discussed in Chapter 9;
- Matrix-assisted laser desorption ionization—time of flight (MALDI-TOF) mass spectrometry (microbial identification). This method is discussed in Chapter 9.

17.5.4 Optical spectroscopy

Optical spectroscopy methods utilize light scattering and other optical techniques to detect, enumerate, and identify microorganisms (e.g., "real time" airborne particle counters). These methods include:
- Real-time and continuous detection, sizing, and enumeration of airborne microorganisms and total particles. These methods are applied to the monitoring of cleanrooms. With this technology, air is drawn into an instrument. Particles that pass through a 405-nm diode laser are sized and enumerated using a Mie scattering particle counter. At the same time, particles that contain biological targets, such as nicotinamide adenine dinucleotide (NADH), riboflavin, and dipicolinic acid, will auto-fluorescence as they pass through the laser, and a separate fluorescence detector will record these as biological events.

17.5.5 Nucleic acid amplification

Nucleic acid amplification technologies are those such as PCR-DNA amplification, ribonucleic acid (RNA)-based reverse-transcriptase amplification, 16S rRNA typing, gene sequencing, and other novel techniques. These methods include:

- riboprinting: 16S sequence of rRNA is highly conserved at the genus and species level. This method is discussed in Chapter 9;
- PCR methods for targeting specific microorganisms (millions of copies of the target DNA in a short period of time). This method is discussed in Chapter 9;
- gene sequencing (specific dye labeling). This is variant of the PCR method. With the method, a genetic analyzer separates the fragments by size and a laser detects the fluorescence color from each dye, producing a full gene sequence of the target DNA.

17.5.6 Microelectrical–mechanical systems

Microelectrical–mechanical systems (MEMS) utilize microarrays, biosensors, and nanotechnology to provide miniaturized technology platforms. These methods include:
- Microarrays (DNA chips) so-called "lab-on-a-chip technology," evolved from Southern Blot technology, to measure gene expression (e.g., mycoplasma detection). With this method, DNA is extracted, and PCR performed using primers specific for conserved and species-specific regions the microbial genome. The fluorescently labeled fragments are then hybridized to the microarray chip. The chip contains probes for species-specific targets.

17.6 Selection of rapid microbiological methods

It is important that care must be taken in choosing an RMM or alternative method for a particular application. The method must determine a product's critical quality attribute and adhere to appropriate good manufacturing practice principles and validation requirements [10].

In some ways, the process of introducing an RMM or alternative method does not differ significantly when compared with implementing a conventional method. The key points are ensuring the method is validated and shows acceptable recovery rates or accurate identification does not differ whether rapid or conventional methods are used [11].

When choosing to implement an RMM, it is important to ensure the new method is appropriate for the company's formulations, facilities, and personnel. For example, the introduction of a method with a higher level of sensitivity needs to be aligned with the existing bioburden in raw materials, environment, and finished products.

Guidance for the implementation of rapid methods is available from both the USP and the Ph. Eur.:

- USP <1223>, *Validation of Alternative Microbiological Methods* [12];
- Ph. Eur. 5.1.6., *Alternative Methods for Control of Microbiological Quality* [13];
- Ph. Eur. 2.6.27, *Microbiological Control of Cellular Products* [14].

In addition, the FDA did issue a draft *Guidance for Industry Validation of Growth-Based Rapid Microbiological Methods for Sterility Testing of Cellular and Gene Therapy Products*, defining validation criteria for growth-based rapid or alternative microbiological methods. The document never became a final text, and it was withdrawn in 2015. From an industry perspective, the Parenteral Drug Association (PDA) has published a useful guide for implementation [15].

There are several considerations to be made and steps to be taken for the implementation of RMMs. These are discussed below.

17.6.1 Key considerations

An important consideration is to decide what is wanted from a rapid method and to consider this alongside a cost–benefit analysis. The first step is to consider the following questions:

- what do I want to achieve?
- what budget do I have?
- what technologies are available?
- what technologies are 'mature'? Who else is using them?
- how "rapid" is the rapid method?
- what papers have been published on the subject? Are these "independent"?
- what have regulators said?

The above can form part of a risk–benefit consideration. Risk–benefit analysis should focus on [16]:

- the defined purpose for the test method;

- the type and depth of information required;
- the limitations of the conventional method and what the rapid method might be able to offer.

Next, a more detailed assessment should be undertaken. This includes considering such factors as time, accuracy, and automation.

With time, factors to consider are:

- time taken to prepare the test; is the rapid method faster, equivalent, or slower?
- time taken to conduct the test;
- sample throughput;
- time to result;
- whether there is a reduction in the time taken to conduct complimentary tests;
- whether more or less time is required for data analysis;
- whether results-reporting is simplified or more efficient?

With accuracy, issues to consider include:

- if the rapid method will lead to a reduction in human error;
- if there is a reduction in subjectivity;
- whether the alternative method will detect more accurately in comparison to a conventional method?
- whether there is a need for the rapid method to detect what a cultural method cannot?

Other considerations include:

- if there is a need for the electronic capture of data?
- whether the method needs to be automated?
- if there is a need for connecting apparatus or linking the method to a laboratory information management system (LIMS)?

17.6.2 Internal company obstacles

The conventional microbiological methods currently used are, generally, already approved and provide meaningful data. Consequently, there may be reluctance within companies to change procedures and adopt RMMs. Thus, arguments relating to the benefits of implementing RMMs may need to be explored.

Furthermore, there may be reluctance to adopt RMMs because of the capital investment in equipment, training, and possible adaption of current manufacturing processes as well as the time and cost of the important validation required before use. The financial implications are naturally important considerations, and it is recommended that discussions on whether or not to employ new RMMs should involve multidisciplinary personnel (e.g., senior management, quality unit, microbiology, production, business development, finance, and members of supply chain). With business issues, one of the key concerns is return-on-investment. This can be assessed by considering the following:

- operating costs of the conventional method;
- operating and investment costs of the RMM;
- cost-savings and cost avoidances of the rapid method.

The following questions can help with this step:

- how much will the validation cost?
- how long will the validation take?
- how many personnel will the validation require?
- how many tests will be needed to run for the validation?
- does the validation require a comparison with another (existing) method?
- how will the data be analyzed and reported?

The cost of implementation should not be considered in isolation; the cost–benefit to the business in terms of higher quality assurance, reduced stock inventory, and quicker release of product may generate cost reduction to the business in excess of the cost of implementation. Capital outlay and running costs will depend upon the RMM chosen, and the equipment purchased.

Other aspects that can support a business case include:

- online/at-line systems can result in reduced microbiology testing and finished product release cycle times;
- RMMs can assist in more immediate decisions on in-process material;
- reduced repeat testing and investigations;
- maximized warehousing efficiencies by way of reduced inventory holding;
- reduction in plant downtime/return from shut downs.
- increased production yield—shift to continuous manufacturing;
- maximized analyst output by eliminating waste activity.

17.6.3 Validation

When choosing an RMM, consideration should be given to how it is going to be validated. Any methods that are being adopted need to yield results equivalent to or better than the method currently used that already gives an acceptable level of assurance. In addition, the RMM and the method currently used should be run in parallel for a designated time as a condition of approval.

Validation will be cantered on two key aspects: the assessment of the equipment and an assessment of the materials that the RMM will assess to demonstrate that microorganisms can be recovered from the material under test [17].

The validation strategy should reflect the RMM selected. Some methods that are based on the analytical chemistry will suit validation criteria that include accuracy and precision, specificity, limit of detection, limit of quantification, linearity and range, and ruggedness and robustness. However, microbiology methods do not necessarily lend themselves to this approach to validation (in that not all of these criteria will be applicable), as FDA indicates [18]:

> *While it is important for each validation parameter to be addressed, it may not be necessary for the user to do all of the work themselves. For some validation parameters, it is much easier for the RMM vendor to perform the validation experiments.*

Therefore, the following validation strategy is recommended:

- define the characteristic of the current test that the RMM is to replace;

- determine the relevant measures that establish equivalence of the RMM to the current method. This may require statistical analysis;
- demonstrate the equivalence of the RMM to the current method in the absence of the product sample;
- demonstrate the equivalence of the RMM to the established method in the presence of the test sample.

More specifically, with certain groups of methods, these various validation considerations can be interpreted as:

(a) qualitative methods
- accuracy and precision, a presence absence test = low number of positives of a low microbial count (fewer than 10 CFU);
- specificity = growth promotion test;
- limit of detection = inoculate at fewer than five CFU in both the pharmacopoeia method and the RMM to be tested over several replicates;
- robustness = different variations of the normal test conditions (e.g., different analysts, different instruments, and different reagent lots).

(b) quantitative methods
- accuracy = suspensions at the upper end of the expected range and then serially diluted down and testing alongside the compendial method. The level of agreement should not be less than 70% compared with the compendia test;
- precision = a statistically significant number of replicates should be used. The level of variance should generally be within the 10–15% and should not be larger than that found within the pharmacopoeia method;
- specificity = carried out using a range of microorganisms;
- limit of quantification = the lowest number of microorganisms that can be reliably counted;
- linearity = a directly proportional relationship between the concentration of microorganisms used and those expressed in the RMM;
- range = the results found in precision, accuracy, and linearity can be used here in order to determine the upper and lower limits of detection of the RMM;
- robustness = different variations of the normal test conditions (e.g., different analysts, instruments, and reagent lots).

During the course of validation, deviations from the established criteria may occur. The implications of these will depend upon the seriousness of the issue and the degree of drift from established parameters. The deviation may or may not lead to a recommencing of the validation after an appropriate change has been made. In the most serious cases, the deviation can lead to the abandonment of the qualification and the rejection of the equipment or system. All deviations require a deviation report to be generated. Deviation reports must be reviewed by a competent expert and be accepted by quality assurance.

With the equipment qualification aspect, validation normally begins with the validation plan (VP). The VP is a document that describes how and when the validation program will be executed in a facility. The VP document will cover some or all of the following subjects:

- introduction;

- plan origin and approval;
- derivation;
- scope of validation activities;
- validation objectives;
- VP review;
- roles and responsibilities;
- an overview of activities;
- division of responsibilities;
- system description;
- overview of system;
- overview of process;
- system description;
- validation approach;
- site activities;
- documentation and procedures;
- scope of documentation;
- validation schedule of activities;
- project master schedule;
- references.

From this plan, equipment validation is normally achieved through appropriate installation qualification (IQ), operational qualification (OQ), and performance qualification (PQ) [19]. Here:

- IQ provides documented evidence that the equipment has been provided and installed in accordance with its specification. The IQ demonstrates that the process or equipment meets all specifications, is installed correctly, and all required components and documentation needed for continued operation are installed and in place;
- OQ provides documented evidence that the installed equipment operates within predetermined limits when used in accordance with its operational procedures;
- PQ provides documented evidence that the equipment, as installed and operated in accordance with operational procedures, consistently performs in accordance with predetermined criteria and thereby yields correct results for the method.

17.6.4 Method transfer

If a validated method is transferred to another laboratory (including third parties), then appropriate change management should be in place. Full validation of the equipment (IQ, OQ, PQ) will need to be carried out. Full validation of the method may not be required, but, as a minimum, it needs to be demonstrated that the method gives equivalent or comparable results to the original laboratory. Any changes to formulations need to be assessed to determine if full or partial revalidation of the method is required [20].

17.6.5 Training

It is important that when RMMs are introduced, sufficient training is provided to ensure a successful and complete implementation of the new methods. This should include the microbiologists and other personnel involved in the running of the tests

and should also take account of the laboratory or manufacturing facilities. Different rapid methods may also require different steps for sample preparation. Rapid methods that require different preparation steps than traditional methods will require additional training and standard operating procedure (SOP) updates.

Qualified microbiologists will still be required to interpret and manage the data, continue to develop the method, and ensure that correct decisions are made. This should form part of the overall microbial quality management system. Consideration can also be given to how rapid methods can be used within the laboratory or within the facility (such as tools for risk assessment) [21].

17.6.6 Expectations from the vendor

Outside of the suitability of the technology, there are a number of points that need to be satisfied in considering a specific technology, most notably the experience of the vendor itself. The following points can be useful:

- what is the vendor's expertise to date?
- is the vendor in a position to support your validation process?
- does the vendor have the relevant quality management system procedures in place?
- what stage is the vendor at in terms of development? For example, is the company financially sound?
- is the technology known to regulators?
- has the vendor made any product filings to regulators?
- does the vendor supply relevant documentation with the technology? For example, design of documents, providing material standards, and so forth.
- does the vendor provide training to analysts?
- is the vendor in a position to react with a reasonable response time to technical issues?
- how often does the vendor envisage system/software updates, and how will these be handled?

17.7 Summary

This chapter has outlined some of the key considerations to be made when deciding whether to adopt a rapid method and the subsequent selection between the different types of rapid methods that are available. The chapter has not set out to differentiate between different technologies (this itself is a rapidly developing field) but more to offer general advice to those tasked with making the selection and undertaking the work required to qualify the method so that it is available for the laboratory or process area to use.

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