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Methods of Rapid Microbiological Assay and Their Application to Pharmaceutical and Medical Device Fabrication

Hideharu Shintani*

Faculty of Science and Engineering, Chuo University, Tokyo, Japan

***Corresponding author:** Hideharu Shintani, Faculty of Science and Engineering, Chuo University, 1-13-27, Kasuga, Bunkyo, 112-8551, Tokyo, Japan, Tel: +81425922336; Fax +81425922336; E-mail: shintani@mail.hinocatv.ne.jp

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Abstract

There are several well-developed rapid microbiological methods now becoming available that may have useful applications in pharmaceutical and medical devices. They are ATP bioluminescence, fluorescent labeling, electrical resistance, and nucleic acid probes. In choosing to employ rapid methods, the microbiologist should examine their prospective performances against the specific requirements for that sector. Some methods may require expensive equipment and offer full automation, and others represent only a small investment. The regulatory view of these methods is changing and they still officially have not been approved in medical and pharmaceutical area, but it will still be up to the microbiologist to demonstrate that the method chosen is fit for the purpose intended.

Keywords: Rapid microbiological methods; Bioburden, Pharmaceuticals; Medical devices

Introduction

Traditional microbiological methods of detection, enumeration, and identification using mostly culture methods are so often time-consuming and labor-intensive. These practical considerations often limit the extent to which microbiological tests are routinely applied both at the formulation development stage (i.e., preservative screening) and for Microbiological Quality Assurance (MQA). In the latter instance, the inevitable time delay associated with incubation often determines that MQA data are only of retrospective value. Pharmaceutical and medical device production and economic pressures can no longer accommodate this delay [1-3]. Considerable benefit would therefore be gained from the introduction of suitable, more rapid methods of microbiological analysis to the pharmaceutical and medical device sector. For these purposes, this review article has been prepared. The typical requirements for such rapid methods are summarized below:

- Rapid
- Sensitive
- Broad spectrum detection
- Potential for specificity
- Identification
- Viability assessment
- Simple
- Potential for automation
- Reproducible
- Compatible with sample matrices

However, they are not always suited to pharmaceutical and medical devices due to the large difference in the number of contaminating microorganisms. Their procedures of detection may be direct, in which individual microorganisms or populations of organisms are directly observed, or indirect, whereby microbial metabolism, metabolites, or components can be monitored. Some methods may be highly developed with extensive equipment and information support, while others can still be considered to be at relatively early stages of research or currently developed for only a narrow application range. Only a few appear able to meet the challenges of pharmaceutical and medical device microbiology [1-4]. It is also important to remember that the term "rapid" is variously applied to techniques of 5 min to 24 h duration, the definition often reflecting the expectations of the user. However, a method that may be deemed rapid in application with a high bioburden may require an extended enrichment period in situations of lower or injured bioburden.

Useful discussion and comparison of the principal methods can be found in the following works: general aspects [5-11], Adenosine Triphosphate (ATP) bioluminescence [3,10,12-15], fluorescent labeling [4,16-23], electrical resistance [4,24-27], enzyme monitoring [3,9,28], *Limulus amoebocyte* lysate [29-31], nucleic acid probes [1,3,32-37], phage-interaction technology [38-43], and carbon dioxide radiometry [44]. In addition to the methods summarized in Table 2, other techniques have been investigated but have received only modest development. These include electrochemical methods [45,46], electronic particle counting [47], microcalorimetry [9,48,49], biophotometry [50], and flow cytometry [2]. Practical details of these methods potentially applicable to the examination of microorganisms attached to medical devices (bioburden) can be found done in Denyer et al. [51].

Currently available rapid methods and their brief explanation

The development of rapid methods has been largely fed by the food, dairy, water, and medical diagnostics industries and has resulted in a diverse range of methods (Table 1).

Method	Detection Principle
Direct	
Fluorescent labeling	Stain microorganisms using a viability-indicating fluorophore; direct enumeration, usually after filter capture, by light excitation (epifiuorescent microscopy or laser scanning) and image analysis
Indirect	
ATP bioluminescence	Light emission from microbial ATP by luciferin/luciferase reaction. Amenable to amplification by intracellular adenylate kinase
Carbon dioxide detection	Monitoring of microbial metabolism using 14C-radiolabeld substrate to produce 14C-labeled carbon dioxide. Infrared CO_2 detection offers a more acceptable substitute
Chromatographic analysis	Detection of microbial metabolites and cellular components; gas chromatographic analysis of microbial fatty acid has been employed in identification
Dye reduction	Monitoring microbial metabolism of specified substrates by color changes in redox dyes; can form the basis of identification profile
Electrical resistance	Measurement of electrical changes (conductance, impedance) in specialized media due to microbial growth; enumeration based on time to exceed a specified detection level
Enzyme monitoring	Detection of microbial enzymes. By using appropriate substrates can form the basis of identification profiles
Limulus amoebocyte lysate	Detection of (principally) Gram-negative bacterial lipopolysaccharide by gelation or colorimetric reaction
Nucleic acid probes	Labeled DNA or RNA probe hybridization to specific target sequences. Amplification of target by the polymerase chain reaction (PCR) increases sensitivity; competitive quantitative PCR offers enumeration
Phage-interaction technology	Host-specific bacteriophage infects target cells leading to phage DNA replication. Detection by expression of new protein (using recombinant phage) or cell lysis

Table 1: A brief explanation of currently available rapid microbiological detection methods

We now recognize that many different types of bacteria, while remaining physiologically active, can enter periods of nonculturability: in this form they are termed Viable but Nonculturable (VNC) [52-54]. This may be an adaptive response to inimical environments; there is even evidence that this characteristic may be the dominant form in some environmental niches [55]. VNC microorganisms are theoretically capable of product spoilage and may be a potential infectious threat [56,57]. It is perhaps reassuring therefore to discover that direct fluorescent staining (labeling) techniques offer a suitable approach to the detection of VNC microorganisms [58]. The ChemScanR process (laser scanning cytometry) routinely shows water bioburdens in excess of those determined by conventional culture, indicative of an otherwise undisclosed VNC population [59].

Use of rapid methods in pharmaceutical and medical devices

From a pharmaceutical and medical device perspective, the principal areas in which rapid methods may find application are given in Table 2.

A method may be required to provide quantitative or qualitative evidence of microbial presence (survival of bioburden), some mechanism of contamination tracking, or to offer rapid confirmation of the absence of microorganisms. Few methods show complete promise in their range and relevance of reported applications (Table 3); some of the practical implications of their use are considered by Newby [4].

Product Quality Assessment

The pharmaceutical and medical device industry has tended to be conservative in its approach to rapid methods for assessment of product quality, largely because of the regulatory constraints imposed upon these products.

Area	Application
Product quality assessment	Microbial limit tests for raw materials and final nonsterile products (includes total viable count and detection of pathogens) Sterility tests
Process hygiene	In-process samples
	Site hygiene
	Air quality
Preservative efficacy	Screening potential preservatives
	Examining the influence of formulation on preservative behavior
Sterilizer testing	Challenge testing
	Biological indicators

Table 2: Principal areas of application for rapid microbiological detection methods in pharmaceutical and medical device production

For this reason, much of the information accumulated in Table 3 is drawn from related industries, but using comparable products and environments. Table 3 clearly demonstrates the current low probability that any single method will satisfy the requirements for all

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types of pharmaceutical and medical device application, although some manufacturers are now seeking methods applicable to a collection of related products. It is unlikely that any rapid method can be immediately applied in a wide range of situations without first undertaking extensive protocol development. The sensitivity of all methods can be enhanced by sample enrichment but this will lead to an inevitable increase in analysis time; additionally, contaminants grow at different rates and this may result in a substantially different microbial flora from the original sampled product. In sterility testing, where the bioburden is quite likely to be low, rapid methods generally require sample enrichment or extended incubation period to reach the microbial levels required for detection, which significantly differs from rapid methods used in the food facilities. Food facilities have approved the use of rapid methods to detect microorganisms contaminating the food products, but pharmaceutical and medical device facilities have not officially approved rapid detection methods yet.

Method	Sensitivity (CFU)	Limitations	Applications	Reference
ATP bioluminescence	>10 yeasts, >10-10 bacteria, reduced to 1-10 range with enrichment or an MPN-based approach.	Presence of high levels of nonmicrobial ATP.	Cosmetics/toiletries	[60]
				[61]
		Interfering factors quenching light or adversely affecting luciferase reaction.	Intravenous fluids	[9,62]
			Medical devices	[63]
			Packaging materials	[64]
			Sterility testing	[65]
			Surface hygiene	[66]
			Water	[67]
Electrical resistance	Threshold for detection -10/mL	Narrow spectrum of detectable organisms without careful media selection; may be overcome with indirect impedance method.	Cosmetics	[68]
			Preservative testing	[69]
			Sterility testing	[70]
			Toiletries	[71]
			Water	[72]
Fluorescent labeling DE(F)T	Air, >10; liquid, generally 10-10/mL but down to 25/mL ; liquid (+ enrichment), 6 organisms irrespective of sample volume	Cannot be applied to highly viscous or particulate materials.	Air	[73]
			Intravenous fluids	[74]
		Direct correlation with viable count not always possible.	Medical devices	[75]
			Preservative testing	[9,76]
			Surface hygiene	[77]
			Water	[78]
			Cosmetics/toiletries	[79]
Laser scanning cytometry	Liquid, to single organism level; in flow cytometry, -50/mL	Spores must be germinated.	Sterility testing	[9,80]
		Viable nonculturable (VNC)organisms may be detected requiring reappraisal of limits.	Water	[81-83]
		Nonfiltable products need to be tested by fiow cytometry	Air	[84,85]
Nucleic acid probes	Better than 0.1 using PCR amplification	Interference from formulation excipients.	Blood products	[71,86]
		Nonviable organisms are also detected limiting utility.	Contamination tracing	[4,58,87]
		Current development focused on specific organisms	Water	[59,88-92]

Table 3: Some examples of rapid methods applied to the detection of microorganisms. DE(F)T: Direct Epifluorescent (Filtration) Technique. Examples besides pharmaceutical and medical devices are also included.

Process Hygiene

In general, the examination of in-process product samples can utilize the same methods of rapid analysis as that for raw materials and final products (Table 3). Surface hygiene assessment, using appropriate swabbing or surface sampling techniques, may require an enrichment period if low counts are expected; similarly, large volumes of water or air associated with the process may need to be sampled and concentrated by filtration to ensure a sufficient microbial bioburden before examination.

Preservative Efficacy

The official preservative efficacy test methods require challenge periods of up to 28 days and the introduction of rapid methods in this situation would confer no meaningful benefit. Where rapid methodology can have an important role to play is in the examination of several candidate preservative systems (and their possible permutations of concentration and combination) for use in new or developing formulations. Here, kinetic data from D-value determinations or estimation of growth-inhibitory concentrations can quickly provide a useful indication of preservative or formulation incompatibilities and can be used to compare the relative merits of potential preservative systems. Rapid methods have been applied to preservative evaluation [74,75,93]. It is important to distinguish between those methods used primarily to explore bacteriostatic behavior and those able to examine bactericidal activity. In the latter instance, enrichment or extended incubation periods may be necessary to detect low numbers of survivors (bioburden), thereby extending the overall detection time.

In a product challenge designed to explore the capacity of a preservative system to withstand repeated microbial insults or to study the ability of spoilage organisms to survive and grow, kinetic information is of less importance and the detection methods summarized in Table 3 are potentially applicable.

Performance of Rapid Method				
Assay Feature	**ATP**	**FL**	**ER**	**NAP**
Rapidity	30 min-24 h	-2 h	1-24 h	4-5 h
Sensitivity (CFU)	1-100	1-200	106-10	(with PCR) 0.1
Broad spectrum	+	+	+	-
Specificity	-	-	+/-	-
Viability assessment	+	+	+	-
Simplicity	+	+	+	+/-
Automation	+	+/-	+	+/-
Sample compatibility	+/-	+/-	+	+/-

Table 4: Current performance of selected rapid methods

Sterilizer Testing

Sterilization protocols require regular microbiological validation; for some processes, continual efficacy monitoring with biological indicators is necessary, which is required in the sterilization validation and GMP. To ensure that every reasonable opportunity is given for the recovery of stressed and injured indicator spores, sometimes a longer incubation period, often in excess of two weeks, is required before assurance of sterilizer efficacy can be given; this provides little opportunity for early detection of partial sterilizer failure. A commercial detection system in which a spore enzyme, α-glucosidase (reflective of spore viability), converts a nonfluorescent substrate into a fluorescent product within an hour offers one solution. Other approaches examined include ATP bioluminescence to detect spores surviving suboptimal sterilization processes [94] and in vivo bioluminescence as a reporter of recombinant spore viability [14].

Conclusion

There are several well-developed rapid microbiological methods now becoming available that may have useful applications in pharmaceutical and medical devices; of these, ATP bioluminescence, fluorescent labeling, electrical resistance, and nucleic acid probes appear among the most promising (Table 4).

Inevitably, no single method will satisfy easily all requirements, and further development will be needed to adapt them to the specific demands of the pharmaceutical and medical device situation. In this context, it is encouraging to see the developments in ATP bioluminescence offering increased sensitivity (adenylate kinase amplification [95]), potential specificity (phage lysins [10], phage lysis [43]), and internal calibration against excipient effects (caged ATP [96,97]), while proposed developments using fluorescently labeled antibodies may offer specificity to laser scanning cytometry [89]. It is particularly pleasing to see that in the reference of Wu et al. [43], two technologies-phage interaction and ATP bioluminescence-come together in one application. The revolution in applied DNA technologies, particularly driven by medical diagnostics, offers major promise in the future for miniaturized nucleic acid-based detection systems; the recognition of gene families associated with particular microbial characteristics [10] offers a route to the detection of specific deteriogens and pathogens.In choosing to employ rapid methods, the microbiologist should examine their prospective performances against the specific requirements for that sector. Some methods may require expensive equipment and offer full automation, and others represent only a small investment. The regulatory view of these methods is changing and they still officially have not been approved in medical and pharmaceutical area, but it will still be up to the microbiologist to demonstrate that the method chosen is fit for the purpose intended.

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