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Currently Available Rapid Microbial Tests for Translational Medicine

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Abstract

Rapid microbial tests ensure testing in rapid and accurate manners at reasonable costs. The ideal test should achieve detection of certain types of microorganisms that are undetected by the pharmacopoeia-defined sterility tests and its accuracy should be confirmed by regulatory authorities. The use of pharmacopoeia sterility tests is limited since they can detect only microorganisms that are viable only in specified media. A rapid test that can detect viable bacteria and microbes that cannot be cultured and never fails in bacterial detection, should appeal to the regulatory authorities. In the agar medium-culture system employed in the pharmacopoeia-microbial test, the test results may be erroneously false negative when the viable bacteria fail to grow under the defined incubation conditions, when inhibitors are present in the test sample, or when scientific approaches are not available to differentiate negative from false negative results. On the other hand, rapid microbial tests with false positive results due to noise caused by detection principle, measurement device, or non-biologic positive substance, imposes huge industrial challenges. In this review, we discuss the rapid microbial tests classified according to various systems for detection of microorganisms and their application, and then introduce some commercial applications of existing devices for detection of microorganisms and provide details of microbial test features, and compare the purposes of the microbial tests.

Keywords: Rapid microbial tests; Translational medicine; Pharmacopoeia

Introduction

Rapid microbial tests ensure testing in rapid and accurate manners at reasonable costs. The ideal test should achieve detection of certain types of microorganisms that are undetected by the pharmacopoeia-defined sterility tests and its accuracy should be confirmed by regulatory authorities.

Many of the chemically synthesized pharmaceutical products can be sterilized but antibody preparations considered under the category of biologics are usually not released commercially until the sterility test results become available. However, with regard to devices for translational medicine, sterility test results may not be available by shipment release but after the administration of such medicine. This poses challenges regarding quality control that require solution. In such context, the application of rapid microbial tests would be of great help for quality control of devices used for translational medicine, ensuring that sterilized products will be used for administration/grafting to patients (or subjects in case of clinical studies). In this regard, the use of pharmacopoeia sterility test is limited since it can only detect microorganisms that are viable in specified media only. It is desirable to have a rapid test that can detect viable as well as non-culturable microbes within the scope of microbiological development, and that has absolute sensitivity and specificity for bacterial detection. Indeed, Kim et al. [1] reported that various bacteria require the presence of other bacteria for growth. Specifically, the growth of bacteria requires iron-containing siderophores, and only 1% of bacteria produce siderophores while the rest utilize the product. This fact may cast doubt on the sensitivity of tests designed for bacterial detection in which conventional agar medium culture fails short of allowing sufficient detection. In agar medium-culture systems employed in the pharmacopoeia-microbial test, the test results may erroneously be false negative when certain viable bacteria fail to grow in the defined incubation conditions, when inhibitors present in the test sample may affect the test, or when no scientific approaches are available to differentiate false negative results from true negative results. On the other hand, rapid microbial tests used to encounter false positives due to the presence of noise caused by either detection principle, the measurement device, or the presence of non-biological positive substance, present huge industrial challenges. In this review, we will discuss the rapid microbial test by classification of the systems that are currently available for the detection of microorganisms and its application, and then introduce some commercial applications of existing detection systems for the identification of microorganisms. We will also describe various features of the currently available microbial tests and compare the indications for these tests.

Classification of detection system for microorganisms and applications

Microorganism detection tests are divided into two major types: growth-dependent tests exploiting bacterial growth capability and growth-independent tests [2]. The results of the latter tests are available much faster than the former type of tests [3]. In terms of the principle of detection of microorganisms, the detection methods are divided into two categories: direct detection of endogenous fluorescent substances present in the microorganism, while the second involves spiking of fluorescent substances or substrates to be converted by microorganisms into fluorescent substances [4,5]. These types are seen in both growth-dependent and -independent systems. The growth-dependent tests detect chemical byproducts, such as low molecules produced during microorganism growth, such as CO_2, acetic acid, and alcohol [6]. This principle is the foundation of the BacT/Alert system (Sysmex, BioMérieux Co.), the most widely used test in the field of translational medicine. The growth-independent systems include Raman spectroscopy and the Mie scattering method for detecting scattered light on the bacterial cell surface, nucleic acid detection technique that can detect microorganism-specific DNA, and methods for detection of microorganism-specific endogenous non-fluorescent substances [7-9]. **Table 1** summarizes the classification of the currently available systems for the detection of microorganisms.

Table 1 Classification of systems available for the detection of microorganisms.

	Principle of detection	Application to bacteria detection
Growth-dependent test	Endogenous fluorescent substance	- Solid-phase cytometry - Flow cytometry - Bioluminescence/fluorescent method
	Exogenous fluorescent substance	- Solid-phase cytometry - Flow cytometry
	Chemical byproducts	Impedance method
		Gas determination
	Visual observation	Microcolony
Growth-independent test	Endogenous fluorescent substance	- Solid-phase cytometry - Flow cytometry
	Exogenous fluorescent substance	- Solid-phase cytometry - Flow cytometry - Bioluminescence/fluorescent method
	Raman spectroscopy	- Solid-phase cytometry - Flow cytometry
	Mie scattering	-Flow cytometry
	Nucleic acid detection technique	-Nucleic-acid amplification testing
	Endogenous non-fluorescent substance	-Fatty acid analysis
		-Infrared spectrophotometry
		-Mass spectrometry

Table 2 summarizes the systems available for detection of microorganisms and their features classified according to the microorganism/measurement principle, irrespective of microorganism growth. These systems are described in detail below.

Table 2 Details of currently available systems for the detection of microorganisms.

Detection system for microorganisms	Description
Solid-phase cytometry	Detects signals emitted by bacteria trapped on a filter by fluorescence and laser microscopes
Flow cytometry	Detects direct signal emitted by bacteria passing through a flow system
Immunological procedure	Detects antibody to specific target bacteria. Largely applying the principle of immunochromatography for detection

Nucleic-acid amplification Testing	Involves amplification/detection of specific using primers specific to certain types of bacteria
Bioluminescence/fluorescent method	Detection of ATP and NADH inside the bacteria
Microcolony method	Detects bacterial microcolonies during the initial phase of colony formation
Impedance Method	Exploits changes in the levels of electric resistance and conductivity associated with increases in metabolites produced by medium ingredients during bacterial proliferation
Gas determination	Exploits changes in gases levels (e.g. carbon dioxide production and oxygen consumption) associated with bacterial growth.
Fatty acid analysis	Bacterial strain identification device. Employs GC-MS. Entails complex pre-treatment. Low precision identification capability. Since the advent of Mass Spectrometry, the method has become obsolete in the bacterial industry.
Infrared spectrophotometry	Bacterial strain identification device. Employs FT-IR used for differentiating bacteria from foreign matter. Non-destructive bacterial strain identification exploiting near infrared radiation
Mass spectrometry	Bacterial strain identification device, using TOF-MS

Currently Available Rapid Microbial Tests

Solid-phase cytometry

The method is based on the principle of trapping bacteria present in liquids by filtration, followed by staining and visualization of the trapped viable bacteria [10]. The staining substrates used for staining viable bacteria exploit the membrane permeability of the target microorganisms, and once they enter the bacterial cells, the fluorescent substrate is disintegrated by enzymes present in the viable bacteria, and fluorescent pigments are released or the fluorescent peak of the substrates undergoes a shift due to loss of membrane permeability of the fluorescent substance with consequent accumulation of substrates within the cells, allowing detection of the microorganism. With regard to dead bacteria, the enzymatic activity or the integrity of bacterial membrane is completely lost, and thus fluorescent substances are not formed or released from the bacterial cell and hence such bacteria are not detected. The method does not require bacterial culture and thus is highly rapid and can deliver the test results within a few hours. These features may present huge advantages for devices available for translational medicine, which cannot be sterilized by the time of shipment release. Notifications pertaining medical devices manufactured for translational medicine propose avoiding the use of antibiotics, meanwhile the use of antibiotics is impermissible in the initial phase of the manufacturing process since raw ingredients, e.g. human-derived cells and tissues, cannot be sterilized. In sterility tests, concerns are raised over possible impacts of antibiotics among other inhibitors on the test system. In case of solid-phase cytometry, small-molecular size agents, such as growth inhibitors, can be filtrated during the process of capturing bacteria by filters, and thus will not affect the test system. As such, the method allows the user to ignore any impact of inhibitors, and it achieves rapid testing by bacterial counting and sterility tests in quantitative and qualitative manners. Since the use of lasers for scanning during determination can damage or kill bacteria, the method is not suitable for identification of bacterial strains.

The approval dossier, including a sterility test that employs solid-phase cytometry method, requires assessment of extrapolation of solid-phase cytometry to pharmacopoeia, in addition to acceptance of the proposed validation. Validation can be conducted visually after measurement using fluorescence microscopes, and there is a precedent for approval by the FDA as an alternative sterility test. The introduction of solid-phase cytometry should bring forward the availability of sterility test results before shipment/administration, in contrast to conventional tests where the test results are available only after administration of translational medicine devices. The multipurpose type of solid-phase cytometry can potentially become the standard test in the future.

Flow cytometry

The method is based on staining viable bacteria present in a liquid sample and its detection in the liquid phase [11]. The method can be potentially modified by replacing staining viable bacteria with the use of fluorescent antibodies in FACS for cell identification. The method allows direct viable bacteria counting that does not depend on microorganism growth and can thus provide rapid test results. The possible impact of inhibitors can be overlooked. The detection sensitivity is inferior to solid-phase cytometry (approximately 100 cfu/mL to 1000 cfu/mL). In addition, the method is less feasible as a sterility test (qualitative test) unless it uses cultured samples, where consideration for the possible impact of inhibitors becomes essential. Autofluorescence is seen in all fluorescent-based assays, not just flow cytometry. One advantage of flow cytometry is that autofluorescent cells can be gated and excluded from the analysis. Low-precision devices may fail to count small bacteria, such as *Pseudomonas aeruginosa*, thus warranting a caution for count determination. As with solid-phase cytometry, flow cytometry can damage and kill bacteria because it employs laser-assisted scanning for bacterial counting and is thus inappropriate for identification of bacterial strains.

In future, development of a device with detection sensitivity of ≥1 cfu/mL may raise expectations for development of translational medicine as less expensive alternative method

instead of detection by solid-phase cytometry. So far, no device based on flow cytometry has been filed for approval as a sterility test.

Immunological procedures

This method incorporates the principle of fluorescent detection and can provide the results rapidly. This method is the best approach to detect specific bacterial strains in the presence of numerous other bacterial strains, if we have specific antibodies to the bacterial strains. The procedure is the most cost-effective and can rapidly detect food poisoning bacteria compared with other approaches. Commercial immune-chromatography kits and detectors are available in the market from a number of suppliers in the food industry [12].

Nucleic acid amplification test

The method detects bacteria based on the principle of trapping viable bacteria using a filter, followed by nucleic acid amplification by targeting gene sequence specific to the bacteria. In general, the method can also detect specific DNA sequences of 16s rRNA among bacterial DNAs [13]. The method also detects DNA sequences of 16s rRNA in the DNA of dead bacteria, which probably explain the high false positive results. Minimization of false positivity can be achieved, however, by using DNase in individual filters used for bacterial trapping followed by thermal denaturation, which allows detection of the DNA sequence of 16s rRNA derived from viable bacteria. In addition, reverse transcription of 16s rRNA can be employed for RNA detection, which can potentially improve specificity.

Although controversy exists over the ability of this method to detect all strains of microorganisms, it is noteworthy that the gene sequence of 16s rRNA is well preserved. This method can also simultaneously detect mycoplasma and viruses based on primer design. The method incorporates the principle of nucleic-acid amplification, similar to the PCR method, and thus it requires attention for quantitative bacterial counting, and commercially available devices/reagents fall short of serving as highly qualitative sterility tests. The rapidness and ease of use of the procedure are superior to other systems that are designed on other principles and designed to detect microorganisms.

Bioluminescence/fluorescence method

The method is not predisposed to inhibitors, such as antibiotics, and can detect all bacterial strains [14]. It is susceptible to false positive results in the presence of contaminants by organisms (e.g. cells). This method achieves detection within several seconds to several minutes, but it is primarily intended for detection of viable bacteria in gases and, thus, is used for real-time monitoring of bacterial presence in the operating room. Devices that can identify bacterial strains are commercially available. The majority of environmental bacteria apparently remain VBNC (viable but non-culturable) due to low temperature, poor nutrition, and drug effects, and in fact, bacterial counts using culture media are often relatively higher than those in air samples. VBNC bacteria can contribute to positive results, and challenges remain in the difficulty of harmonizing with the culture medium method.

Microcolony method

This method involves filtration or 3-dimensional reconstruction of samples, inoculating the filtered material in the exclusive culture cassette and then staining the cultured medium for detection [15]. Since the procedure requires a certain culture period, the tested products are not shipped until the sterility test results become available, and cell preparations need to be cryopreserved until shipment. Validation of the measurement results employs the manual culture method. The method is available for use in bacterial counting and sterility tests.

The technique was designed for viable counting tests for water for pharmaceutical use, and thus aerobic culture was conventionally within the scope of detection. In the test for water for pharmaceutical use, this method employs R2A agar plate medium to check whether the bacteria present in the ingredient water is "≥10 cfu/100 mL" or "≥100 cfu/1 mL". Detection of bacteria is followed by bacterial strain identification. When the above viable counting test is used for the sterility test on devices for translational medicine, demonstration of ≥10 cfu/100 mL indicates extreme sensitivity. Since this is a culture-based method, identification test is needed to detect the bacterial strains, and culture is possible in two temperature bands of 32.5°C and 22.5°C, suggesting the superiority of the method. Meanwhile, based on the limited experience/accumulation of knowledge of anaerobic bacterial culture, validation data are needed, such as a list of detectable anaerobic bacterial strains. Another limitation of this method is that it suitable for only samples that require filtering.

Impedance method

The impedance procedure is based on the principle where changes in impedance (electric resistance) generated by bacterial growth are measured to detect viable culturable bacteria and microorganisms [16]. The principle has been applied to a paper-based microbial fuel cell array [17]. Although the rapidness falls short of flow cytometry, this method still stands in prominence when compared with other methods. The method requires more reasonable running costs, and is thus superior relative to other procedures. However, samples are relatively more susceptible to contaminants: for example, in a sample contaminated with human cells, death of the cells alters the impedance, and hence some limitations exist for samples available for this testing by this method. Furthermore, the potential effects of inhibitors, such as antibiotics, cannot be ruled out. Impedance can vary with sample components, thus necessitating a calibration curve for each sample beforehand. When using this method for quantitative bacterial counting, bacteria prepared at graded concentrations should be inoculated, and a

calibration curve has to be generated, making the method cumbersome and complex. However, the impedance method is suitable as a sterility test (qualitative). Validation of the results employs the manual culture method.

Gas determination

This method is based on measurement of the incremental rate of gases, e.g. CO_2, released from cultured viable bacteria to detect bacterial growth [18]. The method requires processing for several hours to several days, which limits its use as a rapid test method. The method involves inoculation of samples using a syringe and a simple operating procedure, and is mainly used in the field of translational medicine. The method was used previously as an alternative to sterility test for regulatory filing by FDA (US) and PMDA (Japan). On the other hand, the procedure is classified as a pharmacopoeia direct method and thus requires examination of the potential impact of inhibitors, such as antibiotics. The authors use BacT/Alert (SYSMEX bioMérieux Co.) commercially available bottle prefilled with antibiotic absorbing beads (antibiotics inhibitory bottle). Antibiotic removal is possible even at the sample pretreatment stage. The fact that growth and gas emission rates vary with the type of microorganism makes the gas determination method useful for sterility tests only but not for bacterial counting (quantitation).

Fatty acid analysis

The principle of this analytical procedure is extraction of lipids from a post-culture colony, which is then measured by GC-MS, and compared to a database to identify the bacterial strain [19]. The method includes highly complex pretreatment procedure and requires a large amount of bacterial samples in mg unit. Thus, the fatty acid analysis method is feasible for neither bacterial counting nor sterility test. Since the advent of mass spectrometry for standardization, this method is currently rarely used.

Vibrational spectroscopy

Recently, vibrational spectroscopic techniques such as FT-IR (Fourier transform infrared) spectroscopy and micro-Raman spectroscopy with excitation in the visible (VIS) or near infrared (NIR) have demonstrated their great potential in the application of microbial identification [20,21]. The IR and Raman spectrum provide a ‘‘spectral finger-print’’ because it gives us chemical information for identification of microorganisms on a molecular level [22]. These classical vibrational spectroscopy could only reveal the contamination of hundreds of bacterial but single cell. Micro-Raman spectroscopy is a versatile tool for the characterization and discrimination of bacterial cells and identification of unknown bacterial cells on a single-cell level without the need for pre-analytical cultivation of the cells [23]. Therefore, this method has the potential for a rapid identification of microbial pathogens for translational research.

Mass spectrometry

Mass spectrometry uses post-culture bacterial colonies and is capable of rapid bacterial determination involving treatment of colonies with trypsin, protein profiling by TOF-MS, and identifying the bacterial strain of the treated colonies by matching with the database [24]. The method involves very simple procedures and running cost of about 10-cent level, though the device station is prohibitively expensive. The method would pose difficulties in use as a sterility test and is infeasible for bacterial counting. On the other hand, mass spectrometry is highly reliable as a bacterial strain identification method, making it a potentially indispensable analytical procedure in the future.

Conclusions

The selection of the most appropriate bacterial detection system is dictated by the purpose of manufacturing control or shipment control. Hence, we summarized in **Table 3** the appropriateness of the different microbial tests designed for the detection of microorganisms by rating these tests using a three-point scale (using the symbols of ○, Δ and ×) with regard to their use for sterility tests (qualitative), bacterial counting (quantitative), bacterial strain identification, and the speed of testing/availability of results. The symbols used in the Table refer to the grading of the system, where ○ denotes the recommended method, Δ represents possible and × indicates unsuitable method. Especially, taking into account the use of devices for translational medicine for deciding on release acceptability, it appears that solid-phase cytometry and gas determination methods seem to be ideal candidates. On the other hand, based on biomedical and technological advances in the development of nucleic-acid amplification testing and bioluminescence/fluorescent methods, co-development with devices and establishment of validation may render these methods as rapid microbial tests compatible with solid-phase cytometry and gas determination methods.

Table 3 Rating of bacterial tests by various bacterial detection systems.

System for detection of microorganisms	Suitable bacterial test			
	Sterility (qualitative)	Colony count (quantitative)	Bacterial strain identification	Rapid test method
Solid-phase cytometry	○	○	×	○
Flow cytometry	×	○	×	○

Immunological procedure	×	×	Δ	○
Nucleic-acid amplification testing	Δ	Δ	×	○
Bioluminescence/fluorescent method	Δ	○	×	○
Microcolony method	Δ	○	×	×
Impedance method	○	Δ	×	Δ
Gas determination	○	×	×	Δ
Fatty acid analysis	×	×	○	×
Infrared spectrophotometry	×	×	○	×
Mass spectrometry	×	×	○	×
○ recommended method; Δ possible method; × unsuitable method				

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