01/2008:50106

5.1.6. ALTERNATIVE METHODS FOR CONTROL OF MICROBIOLOGICAL QUALITY

The following chapter is published for information.

1. GENERAL INTRODUCTION

The objective of this chapter is to facilitate the implementation and use of alternative microbiological methods where this can lead to cost-effective microbiological control and improved assurance for the quality of pharmaceutical products. These alternative methods may also find a place in environmental monitoring.

The microbiological methods described in the European Pharmacopoeia have been used for almost a century and these methods - for enumerating and identifying micro-organisms - still serve microbiologists well. Over the years, these methods have been invaluable to help control and secure the production of microbiologically-safe pharmaceutical products. Nevertheless conventional microbiological methods are slow, and results are not available before an incubation period of typically up to 14 days. Thus the results from the conventional microbiological methods seldom enable proactive, corrective action to be taken.

Alternative methods for control of microbiological quality have been introduced in recent years, and some of these methods have shown potential for real-time or near-real-time results with the possibility of earlier corrective action. These new methods can also offer significant improvements in the quality of testing.

In this informational chapter new microbiological methods offering pharmaceutical applications are described. For each method the basic principle is stated and the benefits and disadvantages of the method are then discussed. Potential uses describe applications that may be envisaged in view of the principles on which the method is based: it is not intended to suggest that actual application has been made. Finally, general considerations for the validation of the method are outlined. These are illustrated by specific examples for each type of method. A detailed validation protocol is given for information at the end of this chapter.

It is not the intention of this chapter to recommend one method over another, nor is it the intention to provide an exclusive or exhaustive list of alternative methods that can be used for pharmaceutical microbiological control. This informational chapter however may be used in the process of choosing an alternative microbiological method as a supplement or as an alternative to conventional microbiological approaches and to give guidance in the process of validating the chosen method. In this rapidly developing field, further methods are likely to appear. The guidance offered in this chapter may be equally applicable to these methods.

There are 3 major types of determinations specific to microbiological tests. These include:

- qualitative tests for the presence or absence of micro-organisms;
- quantitative tests for enumeration of micro-organisms;
- identification tests.

1-1. *QUALITATIVE TESTS FOR THE PRESENCE OR ABSENCE OF MICRO-ORGANISMS*

In conventional microbiological analysis this type of test is characterised by the use of turbidity or other growth-related changes in a culture medium as evidence of the presence of viable micro-organisms in the test sample. The most common example of this test is the sterility test. Other examples of this type of testing are those tests designed to evaluate the presence or absence of a particular type of viable micro-organism in a sample.

1-2. *QUANTITATIVE TESTS FOR ENUMERATION OF MICRO-ORGANISMS*

Membrane filtration and plate count methods are conventional methods used to estimate the number of viable micro-organisms present in a sample. The Most Probable Number (MPN) method is another example of these methods. MPN was developed as a means to estimate the number of viable micro-organisms present in a sample not amenable to direct plating.

1-3. *IDENTIFICATION TESTS*

Biochemical and morphological characterisation of an unknown micro-organism is the classical method of identification used in pharmacopoeial tests. Recently developed methods have streamlined and automated aspects of this identification, especially in the areas of data handling, analysis, and storage. Several new approaches that have been integrated into these methods include biochemical reactions, carbon substrate utilisation, characterisation of fatty acid composition, restriction endonuclease banding patterns and use of 16S rDNA sequence analysis.

2. GENERAL PRINCIPLES OF ALTERNATIVE METHODS

Alternative microbiological methods employ direct and indirect methods of detection; in some instances amplification of the signal is achieved by enrichment methods. In recognition of these differences, and for convenience within this chapter, alternative methods for the control of microbiological quality are divided into 3 categories:

- growth-based methods, where a detectable signal is usually achieved by a period of subculture;
- direct measurement, where individual cells are differentiated and visualised;
- cell component analysis, where the expression of specific cell components offers an indirect measure of microbial presence.

In some instances, these distinctions are artificial but they do enable a working classification to be created.

2-1. *GROWTH-BASED METHODS*

2-1-1. **Early detection of growth**

2-1-1-1. *General critical aspects of methods based on early detection of growth*

Such methods are critically dependent upon microbial growth in order to achieve a detectable number of micro-organisms. For the typically low levels of microbial contamination seen in pharmaceutical products, detection may take 24 h or even more, especially in the case of yeasts and moulds. Increased sensitivity can be achieved with filtered products. In this case, after filtration, the membrane is incubated in the medium and the result is expressed as presence or absence in the quantity corresponding to the filtered volume. These systems, because they use an incubation step in liquid media, do not offer quantitative information but a presence/absence determination in the quantity analysed. Analysis of more than one sample quantity may offer a semi-quantitative estimation (limit test). The major benefit of such methods compared to classical methods frequently resides in the capacity to process simultaneously a large number of samples and potentially to obtain a result in a shorter time.

2-1-1-2. *Electrochemical methods*

Principles of measurement. Micro-organisms multiplying and metabolising in appropriate growth media produce highly charged ionic metabolites from weakly charged organic nutrients leading to the modification of electrical properties in those media. These changes in impedance (measured by conductance or capacitance) are monitored with electrodes included in the culture vessels and in contact with the culture medium. The measurable end-point is the time taken to detect a pre-determined impedance change; the detection time is inversely proportional to the initial inoculum size. For yeasts and moulds, which produce only small changes in electrical impedance, an indirect measurement of conductance using a potassium hydroxide reservoir is commonly used. Direct measurement of capacitance can also be carried out.

Critical aspects. Automated detection with electronic data generation, mapping of the variation of impedance reflecting the growth curve of the micro-organisms, and reduction of the duration of the test to 48 h.

Potential uses. Microbiological assay of antibiotics, efficacy of antimicrobial preservation and presence/absence in the quantity of sample tested when performing total viable aerobic count.

2-1-1-3. *Measurement of consumption or production of gas*

Principles of measurement. Actively multiplying and metabolising organisms utilise appropriate growth media, leading to the production of metabolites or elimination of specific nutrients. In one approach, changes in gaseous head-space composition may be monitored in closed culture vessels by pressure transducers responding to gas production (e.g. CO_2) or gas consumption (e.g. O_2). Other indicators may be employed including colorimetric detection of CO_2.

Critical aspects. For slow-growing micro-organisms such as mycobacteria, the method offers more rapid detection. There is no direct relationship between original microbial burden and detectable end-point. The incubation temperature and the algorithm for data processing significantly affect the results.

Potential uses. Products where slow-growing micro-organisms may be present.

2-1-1-4. *Bioluminescence*

Principles of measurement. Adenosine triphosphate (ATP) is a well-documented marker of cell viability. In this method, ATP needs first to be released from micro-organisms using an appropriate extractant, followed by quantitative assay using the luciferin/luciferase enzyme system, which emits light in proportion to the ATP present. The emitted light is measured with a bioluminometer and is expressed in relative light units (RLU) for bioluminescence in liquid media. The RLU obtained from the sample is compared with a threshold value determined at 2 or 3 times the RLU of the medium used for cultivation or sample suspension. The result is positive if the RLU obtained with the analysed sample exceeds the threshold value. A modification to the method using growth of micro-organisms captured on a membrane by incubation on agar medium employs a charge coupled device (CCD) camera to detect the micro-colonies, and results are expressed as microCFU. This method is quantitative but has a narrow range of linearity.

Critical aspects. If the product sampled has a high level of bacterial contamination (about 500-1000 CFU per sample quantity tested), the detection is rapid (1 h). For low levels of contamination (less than 100 CFU per quantity of sample tested), it is necessary to increase the number of micro-organisms by an incubation step in culture media (liquid or solid agar) for 12-48 h according to the method employed. After this time, in liquid media, one single cell capable of growth will increase from 1 to 1000 and will be detected. The yield of ATP varies from one micro-organism to another, bacteria containing 1-10 fg per cell and fungi around 100 fg per cell and many other factors including the species, the growth phase of the cell, the nutritional status, the cellular stress or the cellular age could affect the ATP content of the cell. Therefore, it is not possible to obtain a count directly from the RLU value. In addition, turbidity and sample colour can affect the reaction by either enhancing the reaction and increasing the level of light output or acting as a quenching agent and lowering the level of light output. Since the reaction is enzymatically based, products which could inhibit or decrease the enzyme activity may interfere. In practice, such interference is rare but must be thoroughly investigated during the validation process. The reaction is also sensitive to the presence of phosphate nucleotides such as ADP or GTP, which interfere by producing ATP in the presence of adenylate kinase. This enzyme is used to increase the sensitivity of some bioluminescence methods: here a 3rd reagent is added containing ADP and new ATP is produced in the presence of adenylate kinase released from micro-organisms.

Potential uses. Testing for efficacy of antimicrobial preservation, presence/absence in the quantity of sample tested when performing total viable aerobic count (bioluminescence in tube or microtitre plate), total viable aerobic count (bioluminescence on membrane), environmental and water monitoring. The method finds applications in filterable and non-filterable products.

2-1-1-5. *Microcalorimetry*

Principles of measurement. Microbial catabolism generates heat which can be accurately measured by microcalorimetry. Heat production can be detected by placing the contaminated sample in a sealed ampoule containing a growth medium and enclosing within a calorimeter. Using sensitive instrumentation microbial growth curves can be established. High bioburdens may be detectable by flow calorimetry.

Critical aspects. Theoretically, this method does not require microbial growth but simply catabolic activity. Nevertheless, a minimum number of micro-organisms are required to give heat output measures above base-line and this is usually achieved by use of an enrichment method.

Potential uses. Test for efficacy of antimicrobial preservation.

2-1-1-6. *Turbidimetry*

Principles of measurement. Microbial growth will lead to detectable changes in medium opacity. This can be accurately quantified by optical density measurement at a specified wavelength. In its simplest form such measurements are performed in a standard spectrophotometer over a wavelength range generally of 420-615 nm. Alternative automated systems employ microtitre plate readers offering continuous readout with early detection of optical density change.

Critical aspects. Attempts have been made to extrapolate the initial bioburden from the time for detection but this may be limited to healthy micro-organisms with reproducible growth characteristics. The methods cannot distinguish between viable and non-viable micro-organisms.

Potential uses. By means of calibration graphs, determination of the inoculum size of microbial suspensions for use in pharmacopoeial tests. In automated mode, establishment of the preservative sensitivity of test micro-organisms recovered from formulated products.

2-1-1-7. *Phage-based methods*

Principles of measurement. Bacterial viruses (bacteriophage, phage) can infect host cells causing either lysis or incorporation of their genetic material and expression of novel proteins. Their high level of host specificity can be employed in detection methods which exploit the consequences of infection as an end-point. Such end-points include: plaque formation on a solid lawn of reporter bacteria; detection of intracellular contents released from lysed bacteria (possibly by colorimetric method); or phage-induced effects such as ice nucleation or bioluminescence following infection by genetically modified phage. Fluorescently labelled coliphages can be used for the selective detection of viable *E. coli* in combination with DEFT (see 2-3-3.).

Critical aspects. Phage-based detection can be used in both single and mixed cultures where host specificity allows both detection and identification. Detectable end-points often require a minimum number of target cells to ensure a measurable signal, necessitating enrichment in situations of low bioburden. The viral infection process can be adversely affected by sample composition. In most cases there is a narrow host range which makes it difficult to detect a broad spectrum of microbial contaminants.

Potential uses. These methods are used mainly for research purposes with commercial development aimed principally towards uses in clinical and food microbiology. These methods are likely to be employed for presence/absence determinations of specified micro-organisms.

2-1-2. **Media development to improve detection**

Principles of measurement. Culture media have existed for many years and have been constantly improved. A recent innovation is the appearance of chromogenic substrates which are increasingly used in clinical and food microbiology. The ability to detect the presence of specific enzymes using suitable substrates has led to the development of a large number of methods for the identification of micro-organisms employing manual or automated methods. The incorporation of such substrates into a selective or non-selective primary isolation medium can eliminate the need for further subculture and biochemical tests to identify certain micro-organisms. Consequently, chromogenic liquid or solid culture media are designed to produce specific enzymatic activities for detection and differentiation of micro-organisms. In these particular media, defined substrates are introduced into the formulation and are hydrolysed by the specific cell enzyme of a given bacteria or fungi during growth. These substrates are chosen according to the diagnostic enzymatic activity sought and are linked to coloured indicators.

Critical aspects. The use of innovative media presents several advantages: improved discrimination of colonies in mixed culture, ease of use and ease of interpretation. In addition, response times are shorter because growth and identification of the micro-organism are simultaneous. However, validation of the media must be undertaken carefully to ensure a combination of specificity, selectivity and robustness. The quality of the signal is based not only on the careful choice of the enzymes used as the basis of detection, as these enzymes may be present in different genera, but also on physico-chemical characteristics of the medium such as pH.

Potential uses. Detection of specified micro-organisms such as *E. coli*, coliforms, *Salmonella*, *Staphylococcus* and *Streptococcus* spp.; particular benefit may be found in presence/absence testing. Yeasts can also be detected using chromogenic culture media.

2-2. *DIRECT MEASUREMENT*

2-2-1. **Solid phase cytometry**

Principles of measurement. A membrane filter is used to retain microbial contaminants. Micro-organisms are stained by labelling using a fluorophore as a viability indicator, either before or after filtration. The fluorophore is initially a non-fluorogenic, conjugated substrate that requires intracellular enzymatic activity to cleave the substrate and release the fluorescent moiety. An intact cellular membrane is required to retain fluorophore within the cytoplasm. Laser excitation and automated scanning allows the detection of single, viable fluorescent micro-organisms. Appropriate software permits differentiation of viable micro-organisms from auto-fluorescent particles. The high sensitivity and rapidity of detection permits near-real-time detection of microbial contaminants. Total cell counts can be obtained using a permanently fluorescing stain.

Critical aspects. Metabolically active, fastidious and viable non-culturable micro-organisms can be detected. This may result in reappraisal of the microbial limits established for the samples under evaluation. Spores require initiation of germination to enable detection. Single cell detection may be achievable, but identification is not currently part of the routine test protocol. The use of fluorescent antibody may offer a route to selective detection. False positives may occur from auto-fluorescent particles, which can be difficult to differentiate from micro-organisms.

Potential uses. Rapid and sensitive method for the non-specific evaluation of bioburden. It has found applications in testing pharmaceutical-grade waters.

2-2-2. **Flow cytometry**

Principles of measurement. Fluorophore-labelled micro-organisms can be detected in suspension as they pass through a flow cell cytometer. Where a viability-indicating fluorophore substrate is employed, viable micro-organisms can be differentiated from non-viable particles (see 2-2-1.).

Critical aspects. Flow cytometry may be applied for the microbiological analysis of both filterable and non-filterable materials. Flow cytometric analysis gives near-real-time detection, but it is not as sensitive as solid phase cytometry. To increase sensitivity for use in the pharmaceutical field, it often becomes necessary to add an incubation step in culture media and in that case the method becomes a growth-based method. Analysis of non-filterable samples may require serial dilution to optimise performance, and particulate size can have a significant effect on performance. With the exception of filterability, similar considerations apply to those of solid phase cytometry. Clumping of bacteria can be a problem (e.g. *S. aureus*).

Potential uses. In contrast with solid phase cytometry, this method offers the potential to detect and enumerate the microbial bioburden in materials containing significant levels of particulate matter. If a pre-incubation step is needed, the method becomes a qualitative determination.

2-2-3. **Direct epifluorescent filtration technique (DEFT)**

Principles of measurement. This technique may be considered to be a forerunner of solid phase cytometry. Micro-organisms concentrated by filtration from the sample are stained with a fluorescent dye, formerly acridine orange and now more commonly 4′,6-diamidino-2-phenylindole (DAPI), that may be detected by epifluorescent illumination. Fluorescent vital staining techniques as employed in solid phase cytometry (see 2-2-1.) are amenable to DEFT and fluorescent redox dyes such as 5-cyano-2,3-ditolyltetrazolium chloride (CTC) can be used to highlight respiring cells. Coupled with microscopy, the method allows rapid detection of micro-organisms, the absolute sensitivity depending on

the volume of product filtered and the number of fields of view examined. Semi-automated auto-focusing systems coupled to image analysis have served to improve the utility of this method. A modification to the principle employs sampling using an adhesive sheet which permits collection of cells from surfaces, staining on the sheet and subsequent direct observation under the epifluorescence microscope.

Critical aspects. The distribution of micro-organisms on the membrane affects method robustness. The intensity of fluorescence can be influenced by the staining process and the metabolic status of the micro-organisms. A brief period of culture on the filter surface prior to staining allows microcolony formation; these microcolonies stain readily, can be easily enumerated and are demonstrable evidence of viability. Developments using fluorescence *in situ* hybridisation (FISH) arising from the complementary interaction of a fluorescently-labelled oligonucleotide probe with a specific rRNA sequence offer a route to selective detection.

Potential uses. DEFT is generally limited to low viscosity fluids although pre-dilution or pre-filtration has occasionally been applied to viscous or particulate products. Bioburden monitoring has been successfully achieved in aqueous pharmaceuticals.

2-3. *CELL COMPONENT ANALYSIS*

2-3-1. **Phenotypic**

2-3-1-1. *Immunological methods*

Principles of measurement. Antibody-antigen reactions can be employed to detect unique cellular determinants of specific organisms. These reactions can be linked to agglutination phenomena, colorimetric or fluorimetric end-points offering both quantitative and qualitative detection. Enzyme-linked immunosorbent assays (ELISA) offer simple solid phase methodologies.

Critical aspects. Immunological detection methods depend upon the unique expression of specific identifiers but do not necessarily demonstrate the presence of viable micro-organisms.

Potential uses. Detection and identification of specified micro-organisms.

2-3-1-2. *Fatty acid profiles*

Principles of measurement. The fatty acid composition of micro-organisms is stable, well conserved and shows a high degree of homogeneity within different taxonomic groups. The isolate is grown on a standard medium and harvested. The fatty acids are saponified, methylated and extracted and the occurrence and amount of the resulting fatty acid methyl esters are measured by high resolution gas chromatography. The fatty acid composition of an unknown isolate is compared with a database of known isolates for a possible match and identification.

Critical aspects. The use of fatty acid profiles for microbial identification requires a high degree of standardisation. It is critical for the fatty acid composition of microbial cells that isolates are grown using standard media and standard incubation conditions. Standard conditions for operation of the gas chromatograph must be employed, with frequent runs of calibration standards and known isolates being very important.

Potential uses. Identification or characterisation of environmental and product flora for contaminant tracing and detection of specified micro-organisms.

2-3-1-3. *Fourier transform infrared (FTIR) spectroscopy*

Principles of measurement. A Fourier transformation of the infrared spectrum of whole micro-organisms gives a stable, recognisable pattern typical of the taxonomic groups of micro-organisms. The analysis of the FTIR pattern can be performed in instruments available on the market. The isolate is grown on a standard medium and harvested. Cell mass is transferred to a carrier, and the infrared spectrum is recorded. The Fourier transformation is calculated and the pattern is compared with a database of known isolates for a possible match and identification.

Critical aspects. The use of FTIR-patterns for microbial identification requires a high degree of standardisation. It is critical for the FTIR-pattern of microbial cells that isolates are grown using standard media and standard incubation conditions. The cells must be in the same state of the growth cycle when analysed. Particular attention needs to be paid to the validation process.

Potential uses. Identification or characterisation of environmental and product flora for contaminant tracing and detection of specified micro-organisms.

2-3-1-4. *Mass spectrometry*

Principles of measurement. Gaseous breakdown products released by heating microbial isolates in a vacuum can be analysed by mass spectrometry, providing characteristic spectra. Similarly, intact microbial cells, when subject to intense ionisation under matrix-assisted laser desorption ionisation-time of flight (MALDI-TOF) mass spectrometry, release a distinctive pattern of charged species. Such spectra can be compared with known profiles as a rapid aid to identification.

Critical aspects. Isolates require culture prior to analysis.

Potential uses. Identification or characterisation of environmental and product flora for contaminant tracing and detection of specified micro-organisms.

2-3-1-5. *Biochemical assays based on physiological reactions*

Principles of measurement. These assays are usually preceded by a Gram stain or other early differentiation test to decide on the appropriate testing protocol. Microbial cell suspensions are tested using biochemical test kits. Micro-organisms are known to have particular reactions to these biochemical substances, e.g. utilisation of specific carbon sources. The identification of the culture is done by comparing the biochemical reaction profile with a database. These methods can be performed manually or by automated instruments.

Critical aspects. A pure colony is needed which must not be older than 3 days. The handling of the system is easy but the interpretation of the results can be subjective. Depending on the system used and the micro-organism under investigation, the results can be available quickly.

Potential uses. Identification of environmental and product flora for contaminant tracing and detection of specified micro-organisms.

2-3-2. **Genotypic**

2-3-2-1. *Nucleic acid amplification techniques (NAAT)*

General principles of measurement. NAAT rely on the reiteration of the process of DNA polymerisation, leading to an exponential increase of a specific fragment of the nucleic acid, i.e. the use of the polymerase chain reaction (PCR). In this thermophilic cyclic process a specific DNA fragment is amplified using oligonucleotide primers (see also general method *2.6.21*). RNA can also be amplified by PCR after transcription into cDNA using a reverse transcriptase. This technique is known as reverse transcriptase PCR (RT-PCR). Alternatively, specific RNA-based amplification techniques, for example nucleic acid sequence-based amplification (NASBA) or transcription-mediated amplification (TMA) are available to amplify multiple antisense copies of the RNA

target. Amplified nucleic acid fragments can be analysed by several methods: fragment size analysis; specific sequence analysis; reamplification with a second primer pair; or specific detection by hybridisation with a fluorescent labelled probe. Depending on the choice of analysis the amplification technique can be qualitative, semi-quantitative or quantitative. For identification/characterisation purposes sequence analysis of specific parts of the genome can be used (i.e. 16S or 23S rRNA targets).

General critical aspects. NAAT have many advantages over classical methods for the detection of micro-organisms:

- the methods are highly specific, provided that the primers chosen are specific for a particular micro-organism or group of micro-organisms;
- the procedures are rapid, overcoming the problem of prolonged incubation times;
- the methods are highly sensitive allowing ideally the detection and amplification of one single nucleic acid fragment in the reaction mix.

However, there are numerous practical restrictions to its use:

- the sensitivity of the methods is highly dependent on how successfully the target fragments can be concentrated in the sample;
- the presence of inhibitors of the enzymatic process result in false negative reactions;
- the starting volume of the sample tested is small;
- the procedures are prone to cross-contamination from previously amplified fragments resulting in false positive results.

Depending on the aim, a choice must be made for amplification of an RNA or DNA target. The target choice affects the correlation with viability. The use of DNA as a marker has the disadvantage that dead micro-organisms also contain DNA, whereas mRNA is rapidly degraded in dead bacteria and is considered a better marker for viability.

Critical aspects of RT-PCR. Reverse transcriptase-PCR is characterised by the synthesis of cDNA using RNA as a template. Reverse transcriptase is used for this step. A specific part of the cDNA is subsequently amplified by PCR. Depending on the quality of the RNA isolation, the cDNA synthesis efficiency can vary. RT-PCR can be used to specifically detect RNA if the DNA contamination of the RNA sample is minimal.

Critical aspects of RNA amplification techniques. These methods have proven to be very valuable for specific (quantitative) RNA detection. However, they may be more difficult to implement routinely.

Critical aspects of (semi-) quantitative detection (real-time PCR). Classical PCR techniques are based on end-point detection. In general fragment analysis is carried out using agarose gels and specific size markers. However, there is no correlation between the amount of PCR product at the end of the reaction and the original amount of target molecule. In contrast the amount of PCR product detected at the beginning of the exponential phase of the reaction correlates very well with the initial starting amount of nucleic acid. Modern real-time PCR techniques are developed to measure this exponential phase of the reaction. These techniques generate amplification data from which the original amount of target molecule can be deduced. A specific labelled probe detects in real time the PCR product formed, allowing direct visualisation of the exponential part of the PCR reaction. By comparison with amplification plots of a standard dilution series, a quantification of the target molecule can be obtained. Automated real-time PCR systems are available on the market. An additional advantage is that the chance of cross-contamination is minimised, as PCR products are scanned with a laser while the tubes remain closed. However, generation of standards will be difficult to accomplish.

Critical aspects of amplification of genes coding for 16S or 23S rRNA. A powerful application of PCR is the amplification and subsequent sequence analysis of specific parts of the genes coding for 16S or 23S rRNA. Analysis of these specific DNA sequences allows in most cases the identification of a micro-organism at species level. Selection of appropriate universal primers, or even species-specific primer pairs, from international databases allows a high specificity in fragment amplification. Modern systematic classification is based on comparative sequence analysis.

Potential uses. Owing to the high specificity of the amplification techniques, they are very suitable for identification purposes. NAAT are suitable for the detection of specified micro-organisms or certain groups such as mycoplasmas. Real-time quantitative PCR is needed for enumeration.

2-3-2-2. *Genetic fingerprinting*

Principles of measurement. This technique characterises and identifies micro-organisms using restriction fragments of nucleic acids from bacterial and fungal genomes. DNA is extracted from a pure microbial cell lysate and cut into fragments by restriction enzymes. DNA fragments are size-separated by electrophoresis, visualised, and the pattern is compared with other known patterns of microbial isolates. The genetic fingerprint is a stable marker that provides definitive species discrimination or even characterisation below species level. Ribotyping is a typical example of this technique. There are also fingerprinting methods based on PCR with primers that bind to several sites in the microbial genome, creating amplicons with a characteristic size distribution.

Critical aspects. There is a need for a pure colony, but no preliminary cultivation step is necessary. The growth conditions (temperature, type of media,) do not affect the outcome of the analysis. For the identification of bacteria semi-automated systems are on the market.

Potential uses. Genetic fingerprinting is more valuable for strain discrimination (characterisation below species level) than for identification of species.

3. GENERAL VALIDATION REQUIREMENTS

The purpose of this section is to provide guidance on the validation of methods for use as alternatives to microbiological methods of the Pharmacopoeia. For microbial recovery and identification, microbiological testing laboratories sometimes use alternative test methods to those described in the general chapters for a variety of reasons, such as economics, throughput, and convenience. Validation of these methods is required. Some guidance on validation is provided in the General Notices section 1.1 on the use of alternative methods.

Validation of alternative microbiological methods must take into account the large degree of variability associated with conventional methods. When conducting microbiological testing by conventional plate count, for example, one frequently encounters a range of results that is broader than ranges in commonly used chemical tests.

Where specific equipment is critical for the application of the alternative method, the equipment, including computer hardware and software, must be fully qualified as follows:

- design qualification (DQ) to provide documented evidence that the design of the equipment is suitable for correct performance of the method; to be provided by the supplier;

- installation qualification (IQ) to provide documented evidence that the equipment has been provided and installed in accordance with its specification;
- operational qualification (OQ) to provide documented evidence that the installed equipment operates within pre-determined limits when used in accordance with its operational procedures;
- performance qualification (PQ) to provide 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. This is typically done with a 'model' system (with test micro-organisms) to make sure that the conditions used by the user laboratory make it possible to satisfy the criteria described by the supplier of the method in the laboratory.

Some alternative methods depend on the use of databases. The extent of coverage of the database with respect to the range of micro-organisms of interest must be taken into account for validation purposes.

The value of a new or modified method must be demonstrated in a comparative study between the official method and the alternative method. The characteristics defined in this chapter must be used to establish this comparison.

3-1. *TYPES OF MICROBIOLOGICAL TESTS*

It is critical to the validation effort to identify the portion of the test addressed by the alternative method. For example, there are a variety of methods available to detect the presence of viable cells. These methods may have applications in a variety of tests (e.g. bioburden, sterility tests,) but may not, in fact, replace the critical aspects of the test entirely. For example, a sterility test by membrane filtration may be performed according to the pharmacopoeial procedure up to the point of combining the processed filter with the recovery media, and after that the presence of viable cells might then be demonstrated by use of some of the available methods. Validation of this application would, therefore, require validation of the recovery system employed rather than the entire test.

General concerns. Validation of a microbiological method is the process by which it is experimentally established that the performance characteristics of the method meet the requirements for the intended application. Since microbiological tests have 3 basic applications, 3 separate sets of validation criteria are required. These concerns are described below.

3-2. *VALIDATION OF ALTERNATIVE QUALITATIVE TESTS FOR THE PRESENCE OR ABSENCE OF MICRO-ORGANISMS*

3-2-1. **Accuracy and precision**

A direct method to show the equivalence of 2 qualitative methods would be to run them side by side and determine the degree to which the method under evaluation shows equivalence to the pharmacopoeial method. An example of this could be the sterility test where this would translate into a comparison of the rate of positive and negative results produced by the alternative method versus the pharmacopoeial method for identical samples. However, in a case such as the sterility test, the low number of failures would required thousands of comparison tests to establish equivalency and thus would be problematic.

A more feasible method for evaluating the precision of an alternative qualitative method compared with a pharmacopoeial method might be to observe the degree of agreement between the two when the procedures are performed repeatedly on different lots of the same product. The accuracy and precision of the alternative method may be expressed as the relative rates of false positive and false negative results between the new method and the pharmacopoeial method using a standardised, low-level inoculum.

The rate of occurrence of false negative results in the presence of the sample for the 2 methods can be estimated using low levels of test micro-organisms. This design is similar to the standard bacteriostasis/fungistasis test; however, the level of micro-organisms inoculated must be very low, for example about 5 CFU per unit. The level of inoculum should ensure a frequency of failure rates high enough to provide a means to compare the 2 methods. The alternative method must provide at least as high a frequency of recovery as the pharmacopoeial method.

3-2-2. **Specificity**

The specificity of an alternative qualitative method is its ability to detect the required range of micro-organisms that may be present in the sample under test. This concern is adequately addressed by growth promotion of the media for qualitative methods that rely upon growth to demonstrate presence or absence of micro-organisms. For those methods that do not require growth as an indicator of microbial presence, the specificity assures that extraneous matter in the test system does not interfere with the test. Where relevant for the purpose of the test, mixtures of micro-organisms are used during validation.

3-2-3. **Limit of detection**

The limit of detection of an alternative qualitative method is the lowest number of micro-organisms in a sample that can be detected under the stated experimental conditions. A microbiological limit test determines the presence or absence of micro-organisms. Due to the nature of microbiology, the limit of detection refers to the number of micro-organisms present in the original sample before any dilution or incubation steps; it does not refer to the number of micro-organisms present at the time of testing.

The 2 methods (alternative and pharmacopoeial) must be assessed by using an inoculum containing a low number of test micro-organisms, for example about 5 CFU per unit, followed by a measurement of recovery. The level of inoculation must be adjusted until at least 50 per cent of the samples show growth in the pharmacopoeial method. It is necessary to repeat this determination several times, as the limit of detection of a test is determined from an appropriate number of replicates (for example not less than 5). The ability of the 2 methods to detect the presence of single organisms can be demonstrated using the χ^2 test.

3-2-4. **Robustness**

The robustness of an alternative qualitative method is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters, and provides an indication of the method's reliability under a variety of normal test conditions, such as different analysts, instruments, batches of reagents and laboratories. Robustness can be defined as the intrinsic resistance to the influences exerted by operational and environmental variables on the results of the microbiological method. Robustness is a validation parameter best suited to determination by the supplier of the method, but if critical parameters are modified by the user their effects on robustness have to be evaluated. Robustness of a qualitative method is judged by its ability to detect the test micro-organisms under the deliberate variations to the method parameters.

3-3. *VALIDATION OF ALTERNATIVE QUANTITATIVE TESTS FOR ENUMERATION OF MICRO-ORGANISMS*

3-3-1. **Accuracy**

The accuracy of an alternative quantitative method is the closeness of the test results obtained by the alternative method to the value obtained by the pharmacopoeial method. Accuracy must be demonstrated across the practical range of the test. Accuracy is usually expressed as the percentage of recovery of micro-organisms by the method.

Accuracy may be shown by preparing a suspension of micro-organisms at the upper end of the range of the test, serially diluted down to the lower end of the range of the test. For example, if the alternative method is meant to replace the traditional plate count method for viable counts, then a reasonable range might be 10^0-10^6 CFU per ml. If it is, instead, a replacement for the MPN method, a much more narrow range may be used. At least 5 suspensions across the range of the test must be analysed for each test micro-organism. If the alternative method is meant to replace the conventional method, it must provide an estimate of viable micro-organisms of not less than 70 per cent of the estimate provided by the pharmacopoeial method.

The protocol used to check the linearity (see 3-3-5.) of the method may also be used to check the accuracy: the suspensions of micro-organisms prepared for the alternative method are counted at the same time using the pharmacopoeial method. Accuracy is demonstrated if the suitability tests show that the slope of the regression line does not differ significantly from 1 and if the y-intercept is not significantly different from 0.

3-3-2. **Precision**

The precision of an alternative quantitative method is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of homogeneous suspensions of micro-organisms under the prescribed conditions. The precision is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements.

At the very least, a suspension of micro-organisms with a concentration usually in the middle of the range is counted several times. The number of replicates is chosen so that the entire test can be carried out during the same working session, i.e. under the same operating conditions and without any change in the suspension of micro-organisms. Other working sessions are then carried out under conditions of maximum variability (different reagents, different operators, different days, etc.). The variance of the results observed in each of the working sessions ('groups') is calculated. If the variances are homogeneous, the variance of the repeatability can be calculated. The inter-group variance of the results is calculated. The variance of the intermediate precision is the sum of the variance of the repeatability and the inter-group variance. The coefficients of variation are then calculated. Generally, a coefficient of variation in the 10-15 per cent range is acceptable. Irrespective of the specific results, the alternative method must have a coefficient of variation that is not larger than that of the pharmacopoeial method.

3-3-3. **Specificity**

The specificity of an alternative quantitative method is demonstrated using a range of appropriate micro-organisms. Where relevant for the purpose of the test, mixtures of micro-organisms are used during validation.

3-3-4. **Limit of quantification**

The limit of quantification of an alternative quantitative method is the lowest number of micro-organisms that can be accurately counted. As it is not possible to obtain a reliable sample containing a known number of micro-organisms, it is essential that the quantification limit is determined from a number of replicates, for example at least 5. The results of the linearity and accuracy studies can also be used. Here, the lowest concentration in the linear range is considered to be the limit of quantification of the method. The limit of quantification must not be a number greater than that of the pharmacopoeial method.

3-3-5. **Linearity**

The linearity of an alternative quantitative method is its ability to produce results that are proportional to the concentration of micro-organisms present in the sample within a given range. The linearity must be determined over the range corresponding to the purpose of the alternative method. A method to determine this would be to select different concentrations of each test micro-organism and conduct several replicates of each concentration. The number of replicates is chosen so that the entire test can be carried out during the same working session. 2 more working sessions are then completed under conditions of maximum variability (different reagents, different operators, different days, etc.). After checking the homogeneity of the variances of the results obtained for each concentration, the regression line is calculated. Linearity is demonstrated if the estimated slope is significant and if the test for deviation from linearity is non-significant.

3-3-6. **Range**

The range of an alternative quantitative method is the interval between the upper and lower levels of micro-organisms that have been determined with precision, accuracy, and linearity using the method as written. The range is determined from studies of precision, accuracy and linearity.

3-3-7. **Robustness**

The robustness of an alternative quantitative method is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability under a variety of normal test conditions, such as different analysts, instruments, batches of reagents and laboratories. Robustness can be defined as the intrinsic resistance to the influences exerted by operational and environmental variables on the results of the microbiological method. Robustness is a validation parameter best suited to determination by the supplier of the method, but if critical parameters are modified by the user their effects on robustness have to be evaluated. Robustness of a quantitative method is judged by its ability to enumerate with statistical relevance the test micro-organisms under the deliberate variations to the method parameters.

3-4. *VALIDATION OF ALTERNATIVE IDENTIFICATION TESTS*

There is a large body of evidence that different methods vary considerably in their ability to identify micro-organisms in pharmacopoeial products. It must be accepted that a method of systematics needs to be internally consistent, but may differ from others in identification of isolates. In other words, identification of an isolate based on biochemical activity may lead to one conclusion, identification by fatty acid analysis to another, identification by DNA analysis may lead to a third, and other methods may lead to alternative conclusions. Microbiological identifications by a particular system flow directly from previous experience with that system, and therefore may well differ from identifications by another system. It is critical that each system provides a consistent identification of isolates from pharmacopoeial products.

3-4-1. **Accuracy**

The accuracy of an alternative identification method is its ability to identify the desired micro-organism to the required taxonomic level and to differentiate it from other micro-organisms present in the sample. It must be demonstrated with a series of test micro-organisms or micro-organisms obtained from a typical sample previously identified by another method.

3-4-2. **Precision**

The precision of an alternative identification method is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of suspensions of test micro-organisms across the range of the test.

3-4-3. **Robustness**

The robustness of an alternative identification method is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters, and provides an indication of its reliability under a variety of normal test conditions such as different analysts, instruments, batches of reagents and laboratories. Robustness can be defined as the intrinsic resistance to the influences exerted by operational and environmental variables on the results of the microbiological method. Robustness is a validation parameter best suited to determination by the supplier of the method, but if critical parameters are modified by the user their effects on robustness have to be evaluated. Robustness of an identification method is judged by its ability to identify consistently the test micro-organisms under the deliberate variations to the method parameters.

4. SPECIFIC VALIDATION REQUIREMENTS

4-1. *BACKGROUND*

Validation is defined in various contexts with some differences, but a consensus definition is to establish documented evidence that a process will consistently achieve what it is intended to do. Hence, in order to perform correct validation of a new method it is critical to understand and define what the procedure is intended to achieve.

2 levels of validation must be envisaged for the application of conventional or alternative microbiological methods. Primary validation of a method is typically performed by the supplier of the new method, whereas validation for the actual intended use, which is a verification of the suitability or applicability of the method in a given situation, must be seen as the responsibility of the user. Before validation for the actual intended use, performance qualification is carried out by the user as described in 3. *General validation requirements*.

Typically, microbiological methods use specific characteristics of micro-organisms as indicators or detection principles for more general questions. The information needed is presence, number, viability, resistance or identity of micro-organisms in a given product or environment.

A given method will usually give an indirect and conditional answer to the questions. For example, the total number and viability of micro-organisms is indicated by the number of micro-organisms able to reproduce under a certain set of conditions for sample preparation, cultivation and incubation. Reproduction in classical microbiology is hence taken as the general indicator for viability. There are other parameters, however, that can be used as an indication of viability. The level of ATP or accumulation or metabolism of substrates in living cells can also be taken as an indicator for viability. The results of different indication methods for viability may not always be identical. Micro-organisms may not be able to reproduce on a given medium, but may still accumulate and metabolise a substrate. Micro-organisms may be unable at a given state of damage to accumulate a substrate, but may still be able to recover and to reproduce.

Another example is the various methods used for identification of micro-organisms. Characterisation of the metabolic pattern of micro-organisms is frequently used for species identification, whereas another method consists of the comparison of DNA sequences. Again, the answer obtained may not be fully coincident for the different identification methods, and while one answer may be appropriate for the construction of a correct phylogenetic correlation tree, another answer may be more useful in the context of pathogenicity or other properties of the differentiated micro-organisms.

4-1-1. **Primary validation**

In order to characterise a specific microbiological method, the principle of detection must be clearly described by the supplier. The method must be fully detailed with respect to the conditions required for application, the materials and equipment needed, and the expected signal. The application principle should be described in a peer-reviewed journal.

The principle of detection must be characterised in a model system and/or with a panel of test micro-organisms, by at least:

- prerequisite treatment of sample or micro-organisms;
- type of response;
- specificity of the response;
- limit of detection;
- range;
- linearity of the response;
- accuracy and precision of the response;
- robustness of the method in a model system;
- limits of suitability.

Once the method has been characterised in this way by the supplier, the principle of detection need not be verified by each user.

4-1-2. **Validation of alternative microbiological method**

4-1-2-1. *Risk-benefit analysis*

For validation of specific alternative microbiological methods it is critical that the purpose of the procedure is precisely outlined. Based on the purpose, the type and depth of information needed must be defined. The information obtained by, and the limitations of, the conventional method and the alternative method must be considered and compared in a risk-benefit analysis.

An alternative method can be justified as being applicable if the information obtained gives a scientifically sound answer to the questions asked in the procedure, and if the limitations of the method are not more severe than the limitations of the conventional method.

4-1-2-2. *Validation for the actual intended use*

The alternative method must be applied in the procedure used and with the samples to be analysed under the responsibility of the user, and must be shown to give comparable results as characterised in a model system by the supplier. Specific questions to be asked where applicable are:

- compatibility of the response with the sample preparation needed for product testing;
- limit and range of detection of the method with regard to sample size and sample availability;
- specificity of the response with regard to the influence of the ingredients of the product;
- linearity of the response with regard to all types of samples to be analysed;

– accuracy and precision of the response with regard to all types of samples to be analysed;
– robustness of the method with regard to all types of samples to be analysed.

Acceptance criteria for the method in routine use will need to be defined as a function of the application and of the validation data.

4-2. *BIOLUMINESCENCE FOR ENUMERATION OF MICRO-ORGANISMS*

4-2-1. **Risk-benefit analysis**

Extensive scientific evidence and use for years supports the capability of the ATP viability marker to detect the same range of micro-organisms as is encountered using standard plating methods. Since this method is growth-dependent, the improvement comparing to the plating methods is the rapidity to obtain a result (from 5 days with the plating methods to 24 h for bioluminescence). It is possible to identify the bioluminescence-detected micro-organisms from the incubation step medium, but it has to be remembered that in a mixed culture some micro-organisms may out-compete others during incubation. This method provides evaluation of samples within 24 h for filterable and non-filterable products (water, in-process control, environmental samples, solid and liquid raw materials, solid and liquid finished products, etc.) and for a large number of samples, when the detection step is automated.

4-2-2. **Validation for the actual intended use**

The method relies upon the detection of ATP from viable micro-organisms. Performance qualification is carried out with test micro-organisms to make sure that under the conditions applied by the user laboratory it is possible to satisfy the criteria described by the supplier for precision, accuracy and linearity (quantitative method), or limit of detection (qualitative and semi-quantitative method) over the range required for the intended use. Following this step, validation proceeds in 3 phases:

– *phase 1*: fertility of the medium in the presence of the product (if an incubation step is performed);
– *phase 2*: search for interferences that may increase or inhibit the ATP production (by addition of an ATP standard solution to the product to test);
– *phase 3*: comparative testing with the pharmacopoeial method.

A detailed example of validation of the bioluminescence method is given at the end of this chapter.

4-3. *CYTOMETRY (SOLID AND FLOW) FOR ENUMERATION OF MICRO-ORGANISMS*

4-3-1. **Risk-benefit analysis**

Extensive scientific evidence supports the capability of this fluorescence viability marker to detect and/or count a wider range of micro-organisms than are encountered using standard plating methods. Cytometry will detect all viable micro-organisms including some that may not be discernable by growth-based methods. Whilst being rapid, the recovery of micro-organisms post-analysis is limited. Thus the further processing of analysed samples for identification would require alternative fluorescent stains or an alternative method. Currently it is not possible to use this method for routine identification of micro-organisms, although basic morphology is readily discernable in solid phase cytometry under fluorescent microscopes. This method provides rapid evaluation of samples and hence allows for a proactive approach to pharmaceutical manufacturing, facilitating building quality into pharmaceutical operations. This method is not growth-dependent and hence all metabolically active micro-organisms will be detected. However, the limit of detection for flow cytometry is currently such that it cannot be used for enumeration by direct examination for most pharmaceutical samples. If pre-incubation is necessary, the estimation becomes semi-quantitative (limit test).

4-3-2. **Validation for the actual intended use**

The method relies upon the detection of a fluorescent signal from labelled micro-organisms.

Performance qualification is carried out to ensure that the instruments perform within their defined operational parameters. This involves the use of fluorescent standards of prescribed intensity and cultures of known type and number of micro-organisms. These tests challenge the quantitative detection system. Reagents and consumables (negative controls) must also be utilised to ensure that the routine test protocol is applicable, and that the quality of the materials used in the test do not contribute to the final result. Pure culture experiments involving test micro-organisms are used to challenge the detection system, and to compare test results with those obtained using standard plate count. Multiple replicates (at least 5) from overnight cultures diluted across a concentration range (e.g. 100 per cent, 75 per cent, 50 per cent, 25 per cent and 10 per cent) must be used to evaluate linearity, accuracy, precision, range, specificity, limit of quantification (quantitative method) and limit of detection (flow cytometry with pre-incubation step). Since cytometry has high sensitivity (solid phase cytometry can detect single cells, whereas flow cytometry is sensitive to a level of around 10-50 cells per millilitre), and detection is not growth based, the linearity of the instrumentation can be tested by comparison of the actual results with the expected value.

Following this step, validation proceeds in 2 phases: validation with respect to the product to be examined and comparative testing. Results of each phase must be evaluated against pre-determined acceptance criteria using positive and negative controls:

– *phase 1*: individual materials to be evaluated by cytometry must be 'spiked' with a defined level of micro-organisms to ensure that the sample preparation process and the samples themselves do not have an impact upon the performance of the detection system; specifically, the sample matrix must not affect detection (i.e. contain endogenous chromophores, auto-fluorescent particles), and in the case of flow cytometry, sample size/dilution and flow rate must be determined for optimal performance;
– *phase 2*: testing must be performed in which the results obtained by cytometry and the pharmacopoeial method are compared; the number of samples and the testing period must be defined in a comparability protocol; the number of samples required will vary, but must be representative of the material evaluation process (i.e. time/number), and must allow for statistical evaluation; all samples must be prepared according to defined procedures and evaluated against selected validation and acceptance criteria, similar to those used for pure culture evaluation.

4-4. *FATTY ACID PROFILES FOR IDENTIFICATION*

4-4-1. **Risk-benefit analysis**

Identification by fatty acid profiles may be more precise than the identification methods based on metabolic profiles in conventional microbiological culture methods. The database is broader than for conventional culture methods. Pre-incubation is needed, but extraction and identification is faster than in biochemical methods and hence, the result is obtained faster. Other modern methods, such as 16S rRNA sequence analysis or genetic fingerprinting, have a similar

broad differentiation range and give a result as fast as this method.

Separation of closely related micro-organisms (e.g. *E. coli* and *Salmonella* spp.) can be difficult by fatty acid profiles. Where the identification of closely related micro-organisms is especially important, other systems may give more precise results. For a given application it is important to specify which types of micro-organisms are most important to be identified. If it is most critical to characterise the correct phylogenetic species of the isolate, DNA sequence-based identification methods will give more reliable results.

Limitations of identification by fatty acid profiles are also seen in the necessity to grow micro-organisms on standardised media under standard temperature conditions and durations of incubation. Micro-organisms that cannot be cultivated on such media cannot be identified.

4-4-2. **Validation for the actual intended use**

Using a range of test micro-organisms and at least 3 replicate determinations in each case, it must be demonstrated that the method yields consistent results.

A significant number of isolates from typical samples to be analysed by the user must be identified, at least 3 times each. The results in each case should be consistent and in accord with those obtained using alternative identification methods. Where a different identification result is found in another identification system, the reason for the difference must be investigated. Where a scientifically plausible explanation exists for the recognition of a different species, a difference between identification systems may be acceptable. In such a case it must be assured that the recognition of the identified species is robust. It must also be assured that the system does not group poorly recognised isolates under one 'species' thereby simulating the repeated isolation of a single species.

4-5. *NUCLEIC ACID AMPLIFICATION TECHNIQUES*

4-5-1. **Risk-benefit analysis**

NAAT are widely used in diagnostics for their precision and rapidity at a relatively low cost (for the analysis, but not for the instruments,) when compared with the traditional methods. Provided that specific validations have been performed, when NAAT are appropriately used, they may offer advantages in some fields in comparison to classical methods; on the other hand classical methods are generally more easily standardisable, need a lower level of technical competence and may have lower costs. Even when NAAT are not more difficult to perform than traditional methods, the interpretation of the results generally needs a high degree of scientific competence.

When used for identification, DNA-based methods cannot discriminate between dead and live micro-organisms. That means that they cannot be directly used on the product but only after passage on a traditional culture medium, thereby losing part of the advantage in rapidity. Moreover, if used directly on the product at the end of the analysis, these methods do not result in a strain to be used in further experiments and may not give advantages when the micro-organisms to be detected are poorly cultivable or stressed. RNA amplification techniques (e.g. RT-PCR) may identify living micro-organisms (but not spores) directly in the products, but in comparison to traditional methods are much more difficult to use routinely. On the other hand, where specific primers are used, identification (or typing) by NAAT is more precise than the traditional methods and in some cases may have other advantages: for instance for the identification of some vaccines (e.g. cholera vaccine, whole cell pertussis vaccine,) their use may substitute for that of specific sera and contribute to reducing the use of animals, or may give a very specific identification where this is presently lacking (e.g. BCG vaccine).

These methods are in general non-quantitative (PCR) or semi-quantitative (real-time PCR), meaning that their results cannot be compared with those of a colony count where an exact enumeration of the micro-organisms present in the sample is requested, but even if colony count has a valence consolidated in time this dogma may not be verified for bacteria which have a tendency to clump (mycobacteria) or are organised in chains or in clusters (streptococci, staphylococci), therefore an accurate standardisation of the semi-quantitative methods may give results of comparable reliability.

4-5-2. **Validation for the actual intended use**

The method is validated according to chapter *2.6.21*. Comparison of conventional and PCR-based methodologies, which differ in sensitivity and specificity, is particularly difficult and may lead to divergent conclusions.

The following example is published for information and not for general application.

Example validation of an alternative method: detailed protocol followed by a laboratory for the implementation of bioluminescence

BACKGROUND

Methods using a pre-incubation step in liquid medium (bioluminescence in tube or microtitre plate) do not offer quantitative information but a presence/absence determination in the quantity analysed. Using more than a single sample quantity, the system may offer semi-quantitative determination (limit test). For example, the classical tested quantity for viable aerobic count on non-sterile products is 0.1 g or 0.1 ml leading to absence in 0.1 g or 0.1 ml, i.e. less than 10 micro-organisms in 1 g or 1 ml for a negative result and more than or equal to 10 micro-organisms in 1 g or 1 ml in case of a positive result. If 0.01 g or 0.01 ml is tested simultaneously, a negative result corresponds to a number of micro-organisms less than 100 in 1 g or 1 ml. The combination between negative for 0.01 g or 0.01 ml and positive for 0.1 g or 0.1 ml permits an estimate of the contamination level of the product to be less than 100 but more than or equal to 10 micro-organisms in 1 g or 1 ml.

As mentioned in section 2., bioluminescence can be used as a quantitative method if micro-organisms are captured on a filtration membrane and later incubated in culture medium (bioluminescence on membrane).

The protocol below describes validation aspects for qualitative, semi-quantitative and quantitative methods.

PERFORMANCE QUALIFICATION OF THE ALTERNATIVE METHOD

Specificity

Screen the method with test micro-organisms appropriate to the method. For example, for microbial aerobic viable count on non-sterile products, use at least the micro-organisms described in chapter *2.6.12* for the fertility of the media in the presence of product. This determination is performed at least 3 times with each micro-organism. Acceptance criterion: all test micro-organisms are successfully detected.

Limit of detection (only for semi-quantitative or qualitative methods)

Prepare a low inoculum (about 5 CFU in the initial sample) of each test micro-organism. Perform the analysis in at least 5 replicates with the pharmacopoeial method and with the bioluminescence method concerned. Acceptance criterion: the ability of the 2 methods to detect the presence of a single micro-organism can be demonstrated using the χ^2 test. Alternative procedure: prepare a series of dilutions of micro-organisms to have a count in the next dilution of about 5 CFU per inoculum (e.g.: 10 CFU/inoculum, 5 CFU/inoculum, 2.5 CFU/inoculum, 1.25 CFU/inoculum, 0.75 CFU/inoculum). Perform the test on 5 independent series of dilutions with the pharmacopoeial method and with the bioluminescence method concerned. Determine the limit of detection for each method. It corresponds to the last dilution where the result is positive for the 5 series. Acceptance criterion: the limit of detection of the bioluminescence method is equal to or lower than that of the pharmacopoeial method.

Limit of quantification (quantitative method)

This can be performed at the same time as the linearity determination. It corresponds to the lowest concentration of the chosen range that satisfies the criteria for linearity, accuracy and precision. Acceptance criterion: the limit of quantification of the bioluminescence method is equal to or lower than that of the pharmacopoeial method.

Precision

Quantitative evaluation. For each test micro-organism, perform at least 5 replicates during the same series including at least the concentration of micro-organisms corresponding to the middle of the range. Perform 3 independent tests. Carry out a statistical analysis to compare the precision of the 2 methods or calculate the coefficient of variation (CV). Acceptance criterion: CV 15 per cent to 30 per cent or precision not different with the risk alpha equal to 5 per cent between the 2 methods. If precision is different, the bioluminescence method is better than the pharmacopoeial method, indicated by a smaller standard deviation.

Qualitative or semi-quantitative evaluation. Use the alternative procedure described for setting the limit of detection and report the frequency of positive results in parallel with the pharmacopoeial method. Acceptance criterion: the frequency of positive results at the detection limit is 100 per cent and this frequency is better than or equal to the pharmacopoeial method.

Linearity

For each test micro-organism, prepare 5 concentrations in the range of the bioluminescence method (range is normally indicated by the supplier). Perform the pharmacopoeial and the bioluminescence methods in parallel. Repeat this test 2 further times to have results on 3 independent tests. Test for linear regression, presence of a slope, and lack of fit with the F test at alpha equal to 5 per cent. If statistical analysis is not possible, calculate the correlation coefficient (R^2) and the slope between the 2 methods. Acceptance criterion: statistical analysis may show linear regression, the presence of a slope and no lack of fit with a risk of 5 per cent. Equation $y = a + bx$ is determined where b is the slope and a the intercept. If no statistical analysis is available, R^2 is at least 0.9 and the slope does not diverge by more than 20 per cent from 1 (b between 0.8 and 1.2). If the linearity is not demonstrated in such a large range, the range can be decreased and linearity demonstrated with only 3 concentrations in place of 5.

Accuracy

Quantitative evaluation. Accuracy can be determined with data obtained in linearity. For each micro-organism use 3 to 5 concentrations within the linear range of the method. Perform statistical analysis (Student's t test at risk 5 per cent) to test the conformity of the estimated slope (value = 1) versus the obtained slope and to test the conformity of the estimated intercept (value = 0) versus the obtained intercept. For example, if the estimated slope is b with a standard deviation $s_{(b)}$ of 0.090 with 5 concentrations of micro-organisms, calculate $t = (b - 1)/s_{(b)}$. For intercept a, with standard deviation equal to $s_{(a)}$, $t = (a - 0)/s_{(a)}$. Compare these values to the Student's t at 5 per cent, for 13 degrees of freedom (3 tests, 5 concentrations). Acceptance criterion: if the t values obtained are less than the Student's t, the method is exact in the applied range. In the case that there is no conformity for the slope (slope different from 1) or for the intercept (intercept different from 0) the method is not exact over the applied range.

Qualitative or semi-quantitative evaluations. Use the alternative procedure described for setting the limit of detection. Calculate the proportion of false negatives for bioluminescence and for the pharmacopoeial method over all tested dilutions. Compare the extent of false negatives for the 2 or 3 concentrations of micro-organisms just under the detection limit (for example 5 CFU/inoculum, 2.5 CFU/inoculum or 1.25 CFU/inoculum) giving a positive result. By definition, the detection limit corresponds to 0 per cent of false negatives. Acceptance criterion: the percentage of false negatives for the bioluminescence method at sample concentrations below the detection limit must be equal to or lower than that of the pharmacopoeial method.

Range

This is the interval between the lowest and the highest concentrations of micro-organisms where linearity, precision and accuracy have been demonstrated.

Robustness

The information is given by the supplier.

VALIDATION FOR THE ACTUAL INTENDED USE

In the example given, there was no need to determine the accuracy and detection limit in the presence of the product. The validation consists of 3 parts, verifying:

- *phase 1*: the fertility of the medium in the presence of the product;
- *phase 2*: the absence of interference from the product that may increase or inhibit ATP production;
- *phase 3*: the testing of the product in parallel with the pharmacopoeial method.

These 3 parts of validation are performed on 3 independent tests using for example at least 2 different batches of product.

Phase 1: fertility of the medium in the presence of the product

If the product has a known high contamination level (more than 500 micro-organisms per gram or millilitre) the incubation step is unnecessary, the micro-organisms can be detected directly. In this case testing the fertility of the medium in the presence of the product is not necessary. However, pharmaceutical products are generally contaminated at a much lower level and growth of the micro-organism is necessary to obtain detection with bioluminescence. It must therefore be proven that the product does not inhibit the growth of micro-organisms under the conditions of the test. In order to do so, separately add inoculum at not more than 100 CFU for each test micro-organism into the portion of medium containing the

product. For bioluminescence in tube or microtitre plate, perform the bioluminescence test. For bioluminescence on membrane, incubate at 30-35 °C or 20-25 °C for 5 days and count the bioluminescent colonies on the membrane. Acceptance criterion: the test is positive (bioluminescence in tube or microtitre plate); the quantitative recovery of the micro-organism is at least 70 per cent (bioluminescence on membrane).

Phase 2: search for interference of the product

The objective is to show that the product does not add stray light or non-microbial ATP (does not lead to false positive result: criterion A) or does not decrease the ATP detection (does not lead to a false negative result: criterion B).

Bioluminescence in tube or microtitre plate

A. Perform the bioluminescence test with the culture broth alone and with the culture broth in the presence of the product. Determine the RLU value for culture broth alone and the RLU value for culture broth in the presence of product.

B. Perform the bioluminescence test with the culture broth alone and the culture broth in the presence of ATP. Determine the response coefficient for ATP concentration in per cent.

Acceptance criterion:

– *criterion A*: the RLU value of culture broth in the presence of product is less than twice the RLU value of culture broth alone (if criterion A is not satisfied, it is necessary to determine a specific threshold for this product);
– *criterion B*: the RLU value of culture broth in the presence of product and ATP is within the interval 25 per cent to 200 per cent of the RLU value of culture broth in the presence of ATP.

Bioluminescence on membrane: perform the complete bioluminescence test to search for interference. Acceptance criterion: the recovery of micro-organisms is greater than or equal to 70 per cent and not more than 200 per cent.

Phase 3: analysis of the product in parallel with the pharmacopoeial method

Perform the test according to the validated method for the product concerned in parallel with the pharmacopoeial method to show the relationship between the 2 methods for the product concerned, on 3 independent tests and using at least 2 different batches. Express the result as positive or negative in a certain quantity (bioluminescence in tube or microtitre plate) or express the count per filtered quantity (bioluminescence on membrane). Acceptance criterion: results must be correlated with the pharmacopoeial method.

01/2008:50107

5.1.7. VIRAL SAFETY

This chapter provides general requirements concerning the viral safety of medicinal products whose manufacture has involved the use of materials of human or animal origin. Since viral safety is a complex issue, it is important that a risk assessment is carried out. Requirements to be applied to a specific medicinal product are decided by the competent authority.

Where the risk of viral contamination exists, complementary measures are used as appropriate to assure the viral safety of medicinal products, based on:

– selection of source materials and testing for viral contaminants;
– testing the capacity of the production process to remove and/or inactivate viruses;
– testing for viral contamination at appropriate stages of production.

Where appropriate, one or more validated procedures for removal or inactivation of viruses are applied.

Further detailed recommendations on viral safety, including validation studies, are provided, in particular, by the *Note for guidance on virus validation studies: the design, contribution and interpretation of studies validating the inactivation and removal of viruses (CPMP/BWP/268/95)* of the Committee for Proprietary Medicinal Products, and the *ICH guideline Q5A: Viral safety evaluation of biotechnology products derived from cell lines of human or animal origin* (including any subsequent revisions of these documents).

Requirements concerning immunological products for veterinary use are dealt with in the monographs *Vaccines for veterinary use (0062)* and *Immunosera for veterinary use (0030)* and related general chapters.

Risk assessment

A risk assessment with respect to viral safety is carried out where materials of human or animal origin are used as ingredients of medicinal products or in the manufacture of active substances, excipients or medicinal products.

The principle of the risk assessment is to consider various factors that may influence the potential level of infectious particles in the medicinal product and factors related to the use of the medicinal product that determine or influence the viral risk to the recipients.

The risk assessment takes into consideration relevant factors, for example:

– the species of origin;
– the organ, tissue, fluid of origin;
– the potential contaminants in view of the origin of the raw material and the history of the donor(s), preferably including epidemiological data;
– the potential contaminants from the manufacturing process (for example, from risk materials used during manufacture);
– the infectivity and pathogenicity of the potential contaminants for the intended recipients of the medicinal product, taking account of the route of administration of the medicinal product;
– the amount of material used to produce a dose of medicinal product;
– controls carried out on the donor(s), on the raw material, during production and on the final product;
– the manufacturing process of the product and its capacity to remove and/or inactivate viruses.

The risk assessment can be based mainly on the manufacturing conditions if these include rigorous inactivation steps (for example, for gelatin etc., and products terminally sterilised by steam or dry heat as described in the general texts on sterility (*5.1*)).