APPROACH TO ALTERNATIVE MICROBIOLOGICAL METHODS

Note: - The following chapter is for information and is not an official requirement

Introduction

This chapter provides guidance on the concept, user requirement selection, evaluation, and use of microbiological methods as alternatives to compendial methods where this can lead to efficient microbiological control and improved assurance for the quality of pharmaceutical products.

The microbiological methods described in Indian Pharmacopoeia effectiveness of antimicrobial preservatives (2.2.2); microbial contamination in non-sterile products (2.2.9); microbiological assay of antibiotics (2.2.10); sterility test (2.2.11) and maintenance, identification, preservation and disposal of microorganisms (2.2.20) are for detection, enumeration and identification of microorganisms. However, these microbiological tests methods are time consuming and slow and in case of Sterility test, it takes 14 days to complete the test. The results from these methods cannot be used to take any corrective action in time.

Alternative microbiological methods have shown potential for real-time or near real-time results with the possibility of earlier corrective action. These new methods, if validated and adapted for routine use, can also offer significant improvements in the quality of testing.

Alternative methods may be used for raw materials, in-process samples, finished pharmaceutical products, for environment monitoring and for utilities such as water, steam and compressed gasses or wherever applicable for testing of microorganisms etc.

In this chapter, alternative microbiological methods for pharmaceutical products are described. For methods available, the basic principle, the advantages and disadvantages of the method are discussed.

It is not the purpose of this chapter to recommend one method/technique 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.

The information herein may be used, however, in the process of choosing an alternative microbiological method as a supplement or as an alternative to pharmacopoeial microbiological methods and to give guidance on validation of the chosen method.

Alternative methods of analysis may be used for control purposes, provided that the methods used are shown to give results of equivalent accuracy and enable an unequivocal decision to be made as to whether compliance with the standards of the monographs would be achieved if the official methods were used. Automated procedures utilizing the same basic principles as the test procedures given in the monograph may also be used to determine compliance. Such alternative or automated procedures must be validated and are subject to approval by the authority competent to authorised manufacturer of substance or product.

In the event of doubt or dispute, the methods of the Pharmacopoeia are alone authoritative and only the result obtained by the procedure given in the pharmacopoeia is conclusive.

1.1 Current Pharmacopoeial Methods

There are three types of determinations in Microbiology methods as described in Pharmacopoeia.

1. Qualitative test for the present/absent of microorganisms
2. Quantitative test where the microorganisms are enumerated on growth medium.
3. Identification methods where the specified organisms are identified based on genotypic, phenotypic and biochemical tests.

1.2 Alternative Microbiological Methods

Alternative microbiological methods employ direct and indirect methods of detection. Amplification of the signal or peak is achieved by the instruments.

Alternative methods for the control of microbiological quality are divided into categories as following:

1.2.1 Growth based methods

1.2.1.1 General critical aspects of methods based on early detection of growth

This method depends on microbial growth to provide an indication of the presence and/or number of micro organisms. Typically low levels of microbial contamination of the products detection may take 24 h or longer. Increased sensitivity can be achieved with filtered products. In this case, after filtration, the membrane filter is incubated in or on the medium and the result is expressed as presence or absence in the quantity corresponding to the filtered volume. These systems, if implemented 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 early detection methods compared to classical methods is often the capacity to simultaneously process a large number of samples and the potential to obtain a result in a shorter time.

The methods described below (1.2.1.2 to1.2.1.5) can be used for quantitative, semi-quantitative and qualitative analysis. These methods are non-destructive and subsequent identification of microorganisms is also possible.

1.2.1.2 Electrochemical methods

Microorganisms 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 such 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 predetermined impedance change; for particular types of microorganisms, the detection time is inversely

proportional to the initial inoculum size. For yeasts and moulds, which only produce small changes in electrical impedance, an indirect measurement of conductance can be used. Direct measurement of capacitance can also be carried out.

There is no direct relationship between the original microbial level and the detectable end-point. This method may be used in microbiological assay of antibiotics, effectiveness of antimicrobial preservatives, and presence/absence of testing.

1.2.1.3 Measurement of consumption or Production of Gas

These methods detect microbial growth either by changes in the electrical properties of a sensor in response to a change in gas composition or by colorimetric changes of a sensor in response to physicochemical changes in the growth medium in contact with that sensor. The systems are based on non-destructive techniques which enable subsequent identification or strain typing of the micro-organisms. Bacteria and/or fungi may be grown in closed containers and continuous monitoring can be performed using automated instruments that measure gas evolution (e.g.CO2) or consumption (e.g. O2) as surrogate markers of microbial growth. Furthermore, the production of metabolites or elimination of nutrients can lead to changes in pH or redox potential. All of these changes can be measured either directly or indirectly as changes in colorimetric or suitable markers in the growth medium.

There is no direct relationship between the original microbial level and the detectable end- point. The incubation temperature, the physiological state and type of micro-organism, the initial load and the algorithm for data processing can significantly affect the results or the time to detection. These methods can be used for presence/absence testing of filterable or non-filterable samples.

1.2.1.4 Bioluminescence

Adenosine triphosphate (ATP) is a well-documented marker of cell viability. In this method, ATP first needs to be released from the micro-organisms using an appropriate extractant, followed by an assay using the luciferin/luciferase enzyme system, which emits light in proportion to the ATP present. The signal-to-noise ratio can be increased by addition of ADP and converting this ADP into released ATP.

Qualitative Test: Micro-organisms are cultivated in liquid medium. The emitted light is measured with a bioluminometer and is expressed in relative light units (RLU) (e.g. bioluminescence in a tube or a well of a microtitre plate). The RLU obtained from the sample is compared with a pre-determined threshold value. The result is positive if the RLU obtained with the analysed sample exceeds the threshold value.

Quantitative Test: Micro-organisms are captured on a membrane and cultivated by incubation on agar medium. Using a charge coupled device (CCD) camera, the ATP released from microcolonies can be detected by light emission and a quantitative determination is possible.

If the sample has a high level of bacterial contamination, the detection is rapid. For low levels of

contamination, it is necessary to increase the number of micro-organisms using an incubation step in culture media (liquid or solid). The yield of ATP varies from one micro- organism to another and can depend on several factors including the species, the growth phase of the cell, the nutritional status, the cellular stress or the cellular age. Additional factors such as turbidity, sample colour or product matrix effects can also influence bioluminescence measurements. Extraction of ATP is generally a destructive process which should be considered with respect to any subsequent need for identification of detected micro-organisms.

This method can be used for presence/absence testing of filterable or non-filterable samples (e.g. final drug products, in-process control samples, media fill), total aerobic microbial count (TAMC), environmental and water monitoring, testing for effectiveness of antimicrobial preservatives.

1.2.1.5 Turbidimetry

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

Attempts have been made to extrapolate the initial microbial contamination from the time to detection, but this is limited to healthy micro-organisms with reproducible growth characteristics.

This method can be used for determination of inoculum size of microbial suspension, microbiological assay of antibiotics and testing for effectiveness of antimicrobial preservatives.

1.2.2. Direct measurement

1.2.2.1 Solid Phase Cytometry

Micro-organisms are stained for viability by exposure to a conjugated, initially non-fluorogenic, fluorophore. An intact cellular membrane is required to retain and accumulate the fluorophore within the cytoplasm. Inside metabolically-active microbial cells, the conjugate is enzymatically cleaved and the fluorescent derivative is released intracellularly. Micro-organisms are collected on a membrane filter either before or after viability staining.

Membrane surfaces retaining vital-stained cells are then scanned by a laser beam and epifluorescent excitation allows the detection of single, viable fluorescent micro-organisms. Appropriate software allows differentiation of viable micro-organisms from auto fluorescent particles. The high sensitivity and rapidity of the method permit detection of microbial contaminants within a few hours. Total cell counts (viable and non-viable) can be obtained using fluorescent staining.

Metabolically active, fastidious and viable non-culturable micro-organisms can all 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 of isolates might not be possible. False positives may occur due to autofluorescent particles that can be difficult to differentiate from micro-organisms. Signal discrimination and enhancement can be aided by microcolony growth.

1.2.2.2 Flow cytometry

Fluorophore-labelled micro-organisms can be detected in suspension as they pass through a flow cytometer. Viable micro-organisms can be differentiated from non-viable particles by use of a viability-indicating fluorophore (see 1.2.2.1). The cell suspension stream is dispersed into a narrow channel and exposed to a laser which excites the fluorophore. Micro- organisms and particles are then counted in different channels depending on whether or not they contain a fluorescent cell.

Direct flow cytometry may be applied to the microbiological analysis of both filterable and non-filterable materials, and after possible enrichment in the case of the low contamination levels. It gives near real-time detection, but is not as sensitive as solid phase cytometry. To increase sensitivity for use in the pharmaceutical field, it is often necessary to add an incubation step in culture media, in which case the method becomes a combination of a growth-based method and a direct detection method. Particle size and number may have a significant effect on performance, and samples may require serial dilution. With the exception of filterability, similar considerations to those in solid phase cytometry apply. Clumping of microorganisms can be a problem.

In contrast to solid phase cytometry, this method offers the potential to detect and enumerate microbial contamination in materials containing particulate matter and if the material cannot be filtered. If a pre-incubation step is needed, the method becomes a qualitative determination.

1.2.2.3 Epiflurescent filter technique

This technique may be considered a forerunner of solid phase cytometry. Micro-organisms, concentrated by filtration of the sample, are stained with a fluorescent dye (formerly acridine orange and now more commonly 4′,6-diamidino-2-phenylindole (DAPI)), that can be detected by epifluorescent illumination. Fluorescent vital staining techniques, 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 with an absolute sensitivity that is dependent 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 of the principle involves sampling using an adhesive sheet (which permits collection of cells from surfaces), subsequent staining on the sheet itself, followed by direct observation using an epifluorescence microscope.

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. Fluorescence is not necessarily an indicator of viability. 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.

Direct Epifluorescent Filter Technique (DEFT) is generally limited to low viscosity fluids, although pre-dilution or pre-filtration has occasionally been applied to viscous or particulate products. Monitoring of microbial contamination has been successfully applied to aqueous pharmaceuticals.

1.2.3 Cell component analysis

1.2.3.1 Phenotypic Techniques

1.2.3.1.1Immunological Methods

Antibody-antigen reactions can be employed to detect unique cellular determinants of specific microorganisms. These reactions can be linked to agglutination phenomena and colorimetric/chromogenic or fluorimetric and other suitable end-points, which offer both quantitative and qualitative detection. Enzyme-linked immune sorbent assays (ELISA) offer simple solid-phase methodologies.

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

This method used for detection and identification of specified micro-organisms.

1.2.3.1.2 Fatty Acid Profile

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 using 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.

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 standardised media and standard incubation conditions. Standard conditions for operation of the gas chromatograph must also be employed, with frequent runs of calibration standards and known isolates being very important.

This method used for identification or characterization of environmental and product microbial contamination (for contaminant tracing and detection of specified micro-organisms).

1.2.3.1.3 Fourier transforms infrared (FTIR) spectroscopy

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 with commercially available instruments. 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.

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 standardised media and standard incubation conditions. The cells must be in the same state of the growth cycle when analysed, and particular attention must be paid to this in the validation process.

This method used for Identification or characterisation of environmental and product microbial contamination (for contaminant tracing and detection of specified micro-organisms).

1.2.3.1.4 Mass spectrometry

Ionised particles released by exposing microbial isolates to a laser 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.

The isolates must be cultured under standardised conditions prior to analysis.

This method is used for identification or characterisation of environmental and product microbial contaminants (for contaminant tracing and detection of specified micro-organisms).

1.2.3.2 Genotypic Techniques

Identification and detection of micro-organisms as well as characterisation of strains belonging to the same species may be achieved by direct detection of nucleotide target sequences that are unique for a particular microbial species or microbial group, and are targets of the genotypic (DNA or RNA-based) detection techniques. These detection techniques may be separated into 3 broad categories: direct hybridisation, nucleic acid amplification and genetic fingerprinting.

1.2.3.2.1 Direct hybridization

DNA probes are short, labelled, single-strand segments of DNA that hybridise with a complementary region of microbial DNA or RNA. The probe or target DNA is usually labelled with either radioactive, fluorescent or chromogenic molecules in order to provide a hybridisation signal. Hybridisation assays include fluorescence in-situ hybridisation (FISH) and microarray-based techniques.

Hybridisation generally requires a large amount of the target DNA for analysis, which may result in lower detection sensitivity. The availability of suitable probes may be limited.

Due to the high specificity of the sequence-based hybridisation reaction, this method may be used for both detection and identification of micro-organisms.

1.2.3.2.2 Nucleic acid amplification technique

This technique relies on the reiteration of the DNA polymerisation process, leading to an exponential increase of a specific nucleic acid fragment. The polymerase chain reaction (PCR) is the most widely used method for target DNA amplification. In this cyclic process, a specific DNA fragment is copied by a thermo stable DNA polymerase enzyme in the presence of nucleotides and oligonucleotide primers, previously designed to flank the target sequence and to hybridise with it (2.8.1).

After PCR, the amplified nucleic acid targets can be analysed using several methods of post-amplification analysis: fragment size analysis in gel electrophoresis, DNA sequencing or specific detection by hybridisation with a fluorescent-labelled probe. Real-time PCR eliminates the need for further post-amplification processing and offers the additional advantage that the likelihood of cross-contamination is minimised.

An important advantage of real-time PCR is the ability to quantify the starting amount of the DNA target sequence in the original sample, in contrast to conventional PCR techniques, which are based on end-point detection.

Due to the high sensitivity and specificity of amplification techniques, they are suitable for both detection and identification of micro-organisms. Real-time PCR is needed for quantitative or semi-quantitative analysis of the target. Besides quantitative determinations, the real-time PCR technique allows simultaneous detection of multiple targets in a single sample, as long as appropriate primers and probes that allow for multiplexing are employed. The sequencing of different genes (e.g.16S rDNA, 23S rDNA, rpoB, Gyr) is best applied to the identification of micro-organisms.

1.2.3.2.3 Genetic fingerprinting

Genetic fingerprinting is the identification of a strain on the basis of its DNA profile (or RNA for RNA viruses). Individual DNA profiles may be different due to genetic diversity between strains of the same species, and the aim of the fingerprinting methods is to discriminate between these strains. The classical genetic fingerprinting technique characterizes microorganisms using restriction fragments of chromosomal DNA from bacterial and fungal genomes.

Different strains from the same species may exhibit different patterns and these differences are referred to as restriction fragment length polymorphisms (RFLPs). As cutting the chromosomal DNA with restriction enzymes generates too many fragment bands to be efficiently and accurately compared, several modifications of the conventional RFLP-based method have been developed. Examples of the kind of technologies used are ribotyping, pulsed-field gel electrophoresis (PFGE) and amplified fragment length polymorphism (AFLP). Several other fingerprinting methods use PCR to selectively amplify defined subsets of DNA restriction fragments from the entire genome, for example random amplified polymorphic DNA (RAPD)

and variable number tandem repeats (VNTR).

All fingerprinting techniques require that the micro-organism is present as a pure culture. Depending on the method, a preliminary enrichment cultivation step may be necessary if a defined quantity or a specific DNA preparation is required for the test.

Genetic fingerprinting methods are mainly used for strain discrimination (characterization below species level). They are a powerful tool for investigating and tracing the source and the spread of microbial contamination.

2. Validation of Alternative Microbiological Methods

2.1 Validation Process

Two levels of validation must be carried out for the application of alternative microbiological methods, namely primary validation and validation for the intended use. The manufacturer of the alternative technology typically performs primary validation of a method, whereas validation for the 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.

Where specific equipment is critical for the application of a method, the equipment, including computer hardware and software, must be fully qualified.

2.1.1 Description of the technique

In order to characterise a specific microbiological method, the principle of detection must be clearly described by the manufacturer. Through primary validation, the method must be fully detailed with respect to the conditions required for application, the materials and equipment needed and the expected signal. The user shall critically review the available information.

2.1.2 Risk-Benefit Analysis

For validation of specific alternative microbiological methods, it is critical that the purpose of the quality assurance procedure is precisely outlined, as this defines the type and depth of information needed. The information obtained by, and the limitations of, the pharmacopoeial method and the alternative method must be considered and compared in a risk-benefit analysis.

The risk level in adopting an alternative method varies depending on the technology considered, the methodology it replaces, the nature of the measurements taken (qualitative, quantitative or identification), the particular product or process attribute being evaluated, the location of the measurement in the manufacturing process chain and various other factors.

Risk analysis tools may be utilised in order to determine which alternative method is to be implemented, to assist in the justification of its implementation or to better understand the impact of implementation on production and/or product quality. An alternative method can be justified for use if the information obtained gives a scientifically sound measure of microbiological quality, and if the limitations of the method are not more severe than those of the pharmacopoeial method.

2.1.3 Primary validation

The manufacturer, using a panel of test micro-organisms appropriate for the intended use, must characterise the principle of detection. Depending on the type of alternative method, relevant validation criteria shall be selected from those listed below:

- Prerequisite treatment of sample or micro-organisms; Type of response;
- Specificity; Detection limit; Quantitation limit; Range;
- Linearity;
- Accuracy and precision;
- Robustness of the method in a model system.

Data management capabilities and may need to have interface with Laboratory Information Management System.

Verification of primary validation data given by manufacturer

The method is verified using the panel of test microorganisms given by the corresponding pharmacopoeial chapter. The alternative method must be applied according to the specified procedure of the manufacturer, without the samples to be analysed under the responsibility of the user, and must be shown to give comparable results as characterised in the model system used by the manufacturer.

2.1.4 Validation for intended use

Validation for the intended use should encompass the entire process, from the decision to change any aspects of a microbiological testing programme to on-going routine use. It should consist of the following phases:

- User requirement specification (URS);
- Design qualification (DQ);
- Installation qualification (IQ);
- Operational qualification (OQ);
- Performance qualification (PQ).

2.1.4.1 User Requirement

Organizations wishing to develop and validate alternative method produce a user requirement specification document. This document should include all critical functions of the technology, critical user interface requirements, space requirements, environmental requirements, operational requirements, and all other important characteristics of an alternative method for the intended use. These requirements will be specific to the company or organization, as well as to the alternative method's intended use, and therefore the requirements should be generated by user.

Instrument Qualification. Most alternative microbiological methods will depend on specific equipment. This analytical equipment is subject to industry standard instrument qualification requirements.

Validation of alternate technologies. The basic rationale for using an alternative methodology is to improve on some aspect of the existing technology of the current compendial method without sacrificing essential characteristics of that technology (e.g., plate count and membrane filtration). The current technology for compendial microbiology methods consists of detection of the growth of viable microorganisms on (or in) a nutrient medium. The alternative technology must be at least equivalent to the current technology in terms of performance for the intended use.

Method suitability. This consideration must address both the technology's suitability to the specific test and the lack of product inhibition and enhancement on the test results.

Suitability of the technology to the specific test. Many compendial microbiological tests have mandated test requirements. The test results are frequently used to determine compliance with finished product specifications, and the specifications are dependent on sample volume or quantity. The alternative technology must be able to satisfy sample volume requirements as required in the general test method. The use of a lesser volume or sample size is not recommended and would need to be fully justified by the user on a case- by-case basis. The alternative technology is considered suitable if it can meet all critical parameters of the compendial test.

Inhibition and enhancement. Specific products may interfere or enhance the signal of different measurement technologies to the specific signal of interest. This component of alternative microbiological method validation (i.e., suitability) must be demonstrated for each product.

Preparation of the User Requirement Specification document. Input from all stakeholders for the alternate microbiological test method is required. These stakeholders may include representatives from the Microbiology, Quality, Regulatory Affairs, and Operations groups, as well as others. The time spent on this step should be considered an investment in reaching a clear understanding of the company's needs before equipment is purchased, which will drive the performance qualification. At minimum, this document should include the following:

- Purpose and intended use (defined need for instrument)
- Description of who will use the equipment
- Operational requirements (data format, user interfaces, and operating environment)
- Constraints (timetables, downtime, maintenance, user skill levels, product compatibility, limit of detection, accuracy, and rapidity)
- Life cycle (development, testing, delivery, validation, training, and obsolesce)
- Capability (turnaround time, test capacity and throughput, and labor requirements)
- Sustainability (consumables, calibration, validation, and preventative maintenance)

The user may need to tailor the specific recommendations in to their particular instrument qualification specifications.

2.1.4.2 Design qualification (DQ)

The DQ provides documented evidence that the design of any associated equipment is suitable for correct performance of the method. Most alternative method systems are based on commercial off-the-shelf equipment. The DQ is most suitably performed, therefore, by the instrument developer/manufacturer. Nevertheless, the user shall verify that the equipment meets the specifications laid down in the URS for the intended application.

2.1.4.3 Installation qualification (IQ)

The IQ provides documented evidence that the equipment has been provided and installed in accordance with its specifications.

2.1.4.4 Operational qualification (OQ)

The OQ provides documented evidence that the installed equipment operates within predetermined limits when used in accordance with its operational procedures.

2.1.4.5 Performance qualification (PQ)

The PQ provides documented evidence that the method, with the equipment installed and operated according to operational procedures, consistently performs in accordance with predetermined criteria and thereby yields correct results for the method. This is typically done with a panel of micro-organisms (e.g. pharmacopoeial test strains, in-house isolates or stressed/slow-growing micro-organisms). This assures that the conditions employed by the user laboratory make it possible to satisfy the criteria described by the manufacturer of the method in the model system used for the primary validation.

Verification for the intended use

The following points, where applicable, should be addressed:

- Compatibility of the response with the sample preparation that the user normally performs for product testing (method suitability 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 product ingredients; Linearity of the response with regard to the types of samples to be analysed;
- Accuracy and precision of the response with regard to the types of samples to be analysed.

Acceptance criteria for the method will need to be defined as a function of the application and the validation data. Since Alternative Microbiological Method may produce sensitive, accurate results, however while using alternative method, acceptance criteria will change in comparison to conventional methods (Quantitative/Qualitative tests), if this is the case, the responsibility lies with the manufacturer/user to produce/show the similarity/equivalence of the results/acceptance criteria while using alternative methods. Since the Acceptance criteria mentioned for

Microbiological quantitative/qualitative tests in IP is conclusive.

2.2 Validation Criteria

The validation parameters generally recommended for qualitative, quantitative and identification microbiological tests. These criteria are described below and summarised in Table 1.

Table1. Validation criteria for qualitative, quantitative and identification tests

Criterion	Qualitative test	Quantitative test	Identification test
Accuracy	-	+	+
Precision	-	+	-
Specificity	+	+	+
Detection limit	+	+	-
Quantitation limit	-	+	-
Linearity	-	+	-
Range	-	+	-
Robustness	+	+	+
Repeatability	+	+	-
Ruggedness	+	+	-
Equivalency	+	+	-

2.2.1 Accuracy

The accuracy of an alternative method is the closeness of the test results obtained by the alternative test method to the value obtained by the compendial method, to be demonstrated across the dynamic (operational) range of the method.

2.2.2 Specificity

The specificity of an alternate qualitative microbiological method is defined as its ability to detect a range of challenge microorganisms specific to the technology. "Range of microorganisms" may be defined as a limited number of microorganisms representing risk to patient or product, microorganisms found in the manufacturing environment and product failures, microorganisms that are appropriate for measuring the effectiveness of the alternative method, and microorganisms that are representative in terms of morphological and physiological attributes appropriate for the method and the product.

2.2.3 Limit of Detection

The limit of detection (LOD) of an alternate microbiological method is defined as lowest number of microorganisms in a defined volume of sample that can be detected, but not necessarily quantified, under the stated experimental conditions. This should be conducted with the quality control organisms mentioned in IP effectiveness of antimicrobial preservatives (2.2.2); microbial contamination in non-sterile products (2.2.9); sterility test (2.2.11) and maintenance, identification, preservation and disposal of microorganisms (2.2.20)

2.2.4 Robustness

A capacity of the method to remain unaffected by small but deliberate variations in method parameters, e.g., reagent volume, incubation time, or ambient temperature providing an indication of its reliability during normal usage. A measure of robustness is not a comparison between the

compendial and alternate methods; rather, it is a necessary component of validation of the alternate method so that the user understands the limits of the operating parameters of the method. The user may rely on data supplied by test method manufacturer.

2.2.5 Limit of Quantification

The limit of quantification (LOQ) of an alternate microbiological method is defined as the lowest number of microorganisms in a test sample that can be enumerated with acceptable accuracy and precision under defined experimental conditions.

2.2.6 Ruggedness

The degree of precision of test results obtained by the analysis of the same samples under a variety of typical test conditions such as different analysts (for example, three), instruments, and reagent lots (the method for demonstration may follow instrument or materials manufacturer recommendations, or it could be based solely on data supplied by test method manufacturer).

2.2.7 Linearity

The ability to produce results that are proportional to the concentration of microorganisms present in the sample within a given range.

2.2.8 Range

The interval between the upper and lower levels of microorganisms that have been demonstrated to be determined with specified accuracy, precision, and linearity.

2.2.9 Repeatability Precision

The degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of the same suspension of microorganisms and uses different suspensions across the range of the test. Also known as "repeatability".

2.2.10 Method suitability

For each new product to be tested using the validated alternate microbiological method, perform the suitability test as described in general test methods effectiveness of antimicrobial preservatives (2.2.2); microbial contamination in non-sterile products (2.2.9); microbiological assay of antibiotics (2.2.10); sterility test (2.2.11) and maintenance, identification, preservation and disposal of microorganisms (2.2.20) using the number of unit and quantities prescribed and the sample preparation appropriate for the product and the required test sensitivity to determine the absence of a product effect that would obscure the signal of the method. Method suitability may be demonstrated using three independent tests. Only the accuracy and precision validation parameters are required for quantitative methods. For qualitative methods, recovery of challenge organisms as indicated in microbial contamination in non-sterile products (2.2.9) and sterility test (2.2.11) is sufficient.

2.2.11 Equivalency

All microbiological tests are performed to enable informed decision making regarding the

microbiological quality of a product, raw material, component, or process step. In this respect, the intended purpose of microbiological tests may be to either evaluate for the presence or absence of microorganisms (as in the sterility test) or to estimate the number of organisms present. The technological means by which microbiological test methods assess microbiological quality and enable a productquality decision may differ from the growth- based means typical of reference methods. The units of measurement (signal) of a microbiological quality assessment performed using alternative microbiological test methods will generally not be a Colony Forming Units (CFU), but rather a different approach to obtaining a cell count estimate. Therefore, the validation of alternative microbial methods should involve two components: (1) equivalence demonstration and (2) analytical method and equipment qualification.