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CONTENTS

APPENDICES

APPENDIX 10 MICROBIOLOGICAL TESTS

10.1 STERILITY TEST pp. 470-476

Change to read:

10.1 STERILITY TEST

The test is designed to reveal the presence, if any, of contamination with viable micro-organisms in pharmaceutical or medical articles intended for parenteral administration or for other sterile applications, which, according to the Pharmacopoeia, are required to be sterile. A satisfactory negative result, however, only indicates that no contaminating micro-organism has been found in the sample examined in the conditions of the test. Nevertheless, because the sample to be tested is randomly selected from the particular batch it represents, it is thereafter assumed that the whole batch passes the sterility test, which is at present the only method available to the various authorities who have to examine a product for sterility.

Alternative procedures or procedural details may be employed to demonstrate that an article is sterile, provided the results obtained are at least of equivalent reliability. Where a difference appears, or in the event of a dispute, when evidence of microbial contamination is obtained by the procedure given in this Pharmacopoeia, the result so obtained is conclusive of failure of the article to meet the requirements of the test.

Test Conditions

Adventitious microbial growth that is transmitted to an article or to inoculated test culture media from the environment during the course of a sterility test invalidates the results of the test. Hence, it is necessary to demonstrate that the proper precautions have been taken to exclude extraneous micro-organisms throughout the test period.

The test should be carried out under aseptic conditions in an area as free from contamination as possible by the use of disinfecting agents, ultraviolet lamps and air filters. Ultraviolet lamps and disinfecting aerosols should not be used during actual testing operation. The test manipulations should be carried out in a clean room (class 10,000) under a laminar flow hood, with operators dressed in sterilized, static-free clothing, including head- and foot-wears. The air pressure in the testing room should be greater than that of the exterior area. The performance of the laminar flow hood should be monitored by particulate count, settle plates, or slit-sampling devices, and the performance of the filters and ultraviolet lamps checked routinely. Regular, simultaneous control test with known sterile preparations are also advisable.

Culture Media

The culture media used for sterility tests for bacteria and fungi should be capable of supporting the growth of a wide variety of micro-organisms, with both aerobic and anaerobic growth characteristics, including the types found in the environment of the manufacturing operations. More than one culture medium will generally be needed to fulfil these criteria.

The following culture media have been found suitable for the test for Sterility. Fluid Thioglycolate Medium is intended primarily for the culture of anaerobic bacteria but will also sustain the growth of aerobic bacteria. Soybean-Casein Digest Medium is intended primarily for the culture of aerobic bacteria but will also sustain the growth of fungi.

A. Preparation

Culture media for the tests may be prepared as described below, or dehydrated mixtures yielding similar formulations may be used, provided that, when reconstituted as directed by the manufacturer or distributor, they have growth-promoting properties equal or superior to those obtained from the formulae given herein. Media are sterilized in an autoclave using a validated process.

I. FLUID THIOGLYCOLATE MEDIUM (FLUID MERCAPTO ACETATE MEDIUM)

L-Cystine	0.5	g
Sodium Chloride	2.5	g
Dextrose Monohydrate	5.5	g
Agar, granulated (moisture content not in excess of 15 per cent)	0.75	g
Yeast Extract (water-soluble)	5.0	g
Pancreatic Digest of Casein	15.0	g
Sodium Thioglycolate (or *Thioglycolic Acid* 0.3 ml)	0.5	g
Resazurin Sodium Solution (0.01 per cent w/v, freshly prepared)	1.0	ml
Water	1000	ml

Mix and heat until solution is effected. Adjust the pH of the solution with *sodium hydroxide TS* so that, after sterilization, it will have a pH of 7.1 ± 0.2. Filter while hot through a filter paper, if necessary. Transfer the medium to suitable containers that provide a ratio surface to depth of medium such that not more than the upper half of the medium has undergone a colour change indicative of oxygen uptake at the end of the incubation period, and sterilize as directed above. If more than the upper one-third of the medium has a pink colour, the medium may be restored once by heating the containers until the pink colour disappears. When ready for use, not more than the upper one-third of the medium in a container should have a pink colour.

Use Fluid Thioglycolate Medium by incubating it under aerobic conditions.

II. ALTERNATIVE THIOGLYCOLATE MEDIUM
(for devices having tubes with small lumina)

L-Cystine	0.5	g
Sodium Chloride	2.5	g
Dextrose Monohydrate	5.5	g
Yeast Extract (water-soluble)	5.0	g
Pancreatic Digest of Casein	15.0	g
Sodium Thioglycolate	0.5	g
(or *Thioglycolic Acid* 0.3 ml)		
Water	1000	ml

Mix and heat until solution is effected. Adjust the pH of the solution with *sodium hydroxide TS* so that, after sterilization, it will have a pH of 7.1 ± 0.2. Filter, if necessary. Place in suitable vessels, and sterilize by steam under pressure (see Steam Sterilization under "Sterilization", Appendix 12). The medium is freshly prepared or heated on a steam-bath and allowed to cool just prior to use. Do not reheat.

Use Alternative Thioglycolate Medium in a manner that will assure anaerobic conditions for the duration of the incubation period.

III. SOYBEAN-CASEIN DIGEST MEDIUM

Pancreatic Digest of Casein	17.0	g
Papaic Digest of Soybean Meal	3.0	g
Sodium Chloride	5.0	g
Dipotassium Hydrogenphosphate	2.5	g
Dextrose Monohydrate	2.5	g
Water	1000	ml

Dissolve the solids with *water*, heating slightly to effect a solution. Cool the solution to room temperature, and adjust the pH with *sodium hydroxide VS* so that, after sterilization, it will have a pH of 7.3 ± 0.2. Filter, if necessary, and dispense into suitable containers. Sterilize as directed above or by a validated filtration process. Incubate under aerobic conditions.

B. Properties and suitability

All the media to be used must comply with the following tests, carried out on each batch before or in parallel with the test on the article being examined.

(1) STERILITY Incubate portions of the media intended mainly for the detection of bacteria at 30° to 35° and those intended mainly for the detection of fungi at 20° to 25° for not less than 14 days or by incubating uninoculated containers as negative controls during a sterility test procedure. No growth of micro-organisms occurs.

(2) GROWTH PROMOTION Inoculate duplicate test containers of each medium with 10 to 100 viable micro-organisms listed in Table 1, and incubate according to the conditions specified for it. The test media are satisfactory if evidence of growth appears within 5 days. This test can be conducted simultaneously with the use of the media for sterility test purposes. However, the sterility test is considered invalid if the sterility of the media or this growth promotion test is not successful.

(3) VALIDATION TESTS FOR BACTERIOSTASIS AND FUNGISTASIS This validation is performed when the test for sterility has to be carried out on a new product or whenever there is a change in the experimental conditions of the test. The validation may be performed simultaneously with the test for sterility of the product to be examined, but before the results of this test are being interpreted. The procedures are as follows:

Table 1 Test Micro-organisms for Growth Promotion and the Validation Tests

Medium	Test Micro-organisms	Incubation Conditions	Incubation Temperature
Fluid Thioglycolate Medium	*Staphylococcus aureus* (ATCC[1] 6538, DMST[2] 8013)*	Aerobic	30° to 35°
	Pseudomonas aeruginosa (ATCC 9027, DMST 15501)**		
	Clostridium sporogenes (ATCC 11437, DMST 15536)†		
Alternative Thioglycolate Medium	*Clostridium sporogenes* (ATCC 11437, DMST 15536)†	Anaerobic	30° to 35°
Soybean-Casein Digest Medium	*Bacillus subtilis* (ATCC 6633, DMST 5871)	Aerobic	20° to 25°
	Candida albicans (ATCC 10231, DMST 5812)		
	Aspergillus niger (ATCC 16404, DMST 15538)		

* An alternative strain is *Bacillus subtilis* (ATCC 6633, DMST 5871).
** An alternative strain is *Micrococcus luteus* (ATCC 9341, DMST 15503).
† An alternative strain is *Bacteroides vulgatus* (ATCC 8482, DMST 15535).
[1] ATCC = American Type Culture Collection
[2] DMST = Department of Medical Sciences, Thailand

Membrane Filtration Method: Filter the test specimen and rinse the membrane with minimum of three 100-ml portions of the appropriate rinsing fluid. Inoculate the final rinse with less than 100 colony-forming units, for each appropriate micro-organism specified in Table 1. Repeat the rinse procedure on another filter that has not been exposed to the specimen under test. This filter will serve as the positive control. Incubate these filters for not more than 7 days under the condition indicated in Table 1 and compare the growth.

If the growth of each test organism in the test containers is visually comparable to the growth in the positive control, use the same amounts of article, number and volume of rinses, and medium when conducting the sterility test. If the growth of the test organisms in the test containers is not visually comparable to that in the positive control, the amount of article used is bacteriostatic or fungistatic. Repeat the test, using a larger number of rinses. Changes in the type of membrane filter used and in the use of neutralizing agents, if available, may reduce the antimicrobial effect of the article. If five rinses, each of about 500 ml, fail to neutralize the antimicrobial residue on the test filter membrane, proceed with the sterility test.

Direct Inoculation Method: Inoculate two containers of each sterility test medium with less than 100 colony-forming units, using the volume of medium (see Table 3) for each appropriate micro-organism specified in Table 1. Add the specified portion of the article under test to one of the inoculated containers of each medium. The other inoculated container is the positive control. Repeat the procedure for each appropriate micro-organism, and incubate the containers at the appropriate temperature for not more than 7 days.

If the growth of the test organisms in the test container is not visually comparable to that of the inoculated control container, the article is bacteriostatic or fungistatic. The use of a sterile neutralizing agent, such as polysorbate 80, lecithin, azolectin, or β-lactamase, may be appropriate. If a neutralizing agent is not effective, establish suitable increased volumes of medium. Use the smallest volume of medium in which the growth of test micro-organisms in the presence of the article is not adversely affected. If the medium volume is increased to 2000 ml and antimicrobial activity is still present, proceed with the sterility test using the 2000 ml of medium. Volumes of medium greater than 2000 ml may be needed for testing medical devices to permit complete immersion of the device.

Sampling of Test Specimens

Unless otherwise directed in the individual monographs, test the number of articles specified in Table 2. If the contents of each article are of

sufficient quantity (see Tables 3 and 4), they may be divided so that equal appropriate portions are added to each of the specified media. If each article does not contain sufficient quantities for each medium, use twice the number of articles indicated in Table 2.

Opening the Containers

Cleanse the exterior surfaces of ampoules and closures of vials and bottles with a suitable decontaminating agent, and gain access to the contents in suitable aseptic manner. If the vial contents are packaged under vacuum, admit sterile air by means of a suitable sterile device, such as a needle attached to a syringe barrel filled with nonabsorbent cotton.

For purified cotton, gauze, surgical dressings, and related Pharmacopoeial articles, open the package or container aseptically.

Quantity of Article

When using the Membrane Filtration Method, unless otherwise specified elsewhere in this chapter or in the individual monograph, use whenever possible the entire contents of each container, but not less than the quantities specified in Tables 3 and 4. When using the Direct Inoculation Method, use the quantities indicated in Tables 3 and 4.

Incubation

Unless otherwise directed in the individual monograph, incubate the test mixture for 14 days with Fluid Thioglycolate Medium or Alternative Thioglycolate Medium, where so indicated, at 30° to 35°, and with Soybean-Casein Digest Medium at 20° to 25°. For products terminally sterilized by a validated moist heat process, incubate the test specimen for not less than 7 days, if the Membrane Filtration Method is used.

Test for Sterility of the Article

The test may be carried out using either Method I, Membrane Filtration, or Method II, Direct Inoculation, as described below, taking into account any modifications described in the appropriate section. Method I is to be preferred whenever the nature of the article to be examined permits, that is,

Table 2 Minimum Number of Articles to Be Tested in Relation to the Number of Articles in the Batch

Number of Articles in the Batch	Number of Articles to Be Tested
Injection / for Injections	
Not more than 100 articles	10 per cent or 4 articles, whichever is greater
More than 100 but not more than 500 articles	10 articles
More than 500 articles	2 per cent or 20 articles, whichever is less
For large-volume parenterals	2 per cent or 10 containers, whichever is less
Antibiotic Solids	
• pharmacy bulk packages (< 5 g)	20 containers
• pharmacy bulk packages (≥ 5 g)	6 containers
• bulks and blends	*See Solid Bulk Products*
Products Not Intended for Injection	
Not more than 200 articles	5 per cent or 2 articles, whichever is greater
More than 200 articles	10 articles
Devices	
Not more than 100 articles	10 per cent or 4 articles, whichever is greater
More than 100 but not more than 500 articles	10 articles
More than 500 articles	2 per cent or 20 articles, whichever is less
Solid Bulk Products	
Up to 4 containers	Each container
More than 4 but not more than 50 containers	20 per cent or 4 containers, whichever is greater
More than 50 containers	2 per cent or 10 containers, whichever is greater

Table 3 Quantities of Article for Liquid Products*

Container Content (ml)	Minimum Volume Taken from Each Product Container for Each Medium	Minimum Volume, in ml, of Each Medium	
		Used for Direct Inoculation of Volume Taken from Each Container**	Used for Membrane or Half Membrane Representing Total Required Volume from the Appropriate Number of Containers
Less than 10	1 ml, or entire contents if less than 1 ml	15	100
10 to less than 50	5 ml	40	100
50 to less than 100	10 ml	80	100
50 to less than 100, intended for intravenous administration	1/2 content	200	100
100 to 500	1/2 content	NA***	100
Over 500	500 ml	NA	100
Antibiotics (liquid)	1 ml	NA	100

* Constitute powder products according to the manufacturer's instructions, and then treat as liquid products.
** For products that cannot be tested by the membrane filtration test procedure.
*** Not applicable

Table 4 Quantities of Article for Solid Products

Container Content	Minimum Quantity Taken from Each Container for Each Medium	Minimum Volume, in ml, of Each Medium	
		Direct Inoculation*	Membrane Filtration
Less than 50 mg	Whole content	200	100
50 mg or more to less than 200 mg	Half the content	200	100
200 to less than 300 mg	100 mg	200	100
300 to 600 mg	200 mg	200	100
More than 600 mg	200 mg	200	100
Antibiotic solids			
for injection (< 5 g)	150 mg	200	100
for injection, pharmacy bulk packages (≥ 5 g)	500 mg	200	100
bulks and blends	See Table 2	200	100
Surgical dressings, cotton, gauze (in packages)	100-mg portion	200	NA***
Sutures and other individually packaged single-use materials	Whole devices	Not more than 2000	NA
Other medical devices	Whole devices (Cut in pieces or disassembled)	Not more than 2000**	NA

* For products that cannot be tested by the membrane filtration test procedure.
** Unless the device is bulky and more than 2000 ml is needed to submerge the device in the medium.
*** Not applicable.

for filterable liquids and for those miscible with, or soluble in, aqueous or oily solvents that do not have an antimicrobial effect under the conditions of the test.

Method I: Membrane Filtration The sterility testing of the articles should be performed when feasible by membrane filtration of the test specimens. This procedure is applicable in the sterility testing of non-bacteriostatic or non-fungistatic liquids or soluble powders, and is particularly appropriate where the article is an oil, an ointment, or a cream that can be put into solution with non-bacteriostatic or non-fungistatic dilution fluids or solvents. The membrane filtration technique is suitable also in the sterility testing of liquids and soluble powders that possess inherent bacteriostatic or fungistatic properties. Certain devices also may be appropriately tested for sterility of the critical pathways by the membrane filtration technique.

Strict aseptic precautions are needed in the manipulations of the tests; the frequent use of negative controls is highly recommended.

Apparatus A suitable unit consists of a closed reservoir and a receptacle between which a properly supported membrane or membranes of appropriate porosity is (are) placed. A membrane generally suitable for sterility testing has a nominal porosity of not greater than 0.45 µm, and a diameter of approximately 47 mm. Cellulose nitrate filters, for example, are used for aqueous, oily and weakly alcoholic solutions and cellulose acetate filters, for example, for strongly alcoholic solutions. The membranes having hydrophobic edges or low product binding characteristics that minimize inhibitory product residue may be needed for certain products, e.g. for antibiotics. The apparatus must be so designed that solution to be examined can be introduced and filtered under aseptic conditions and it must permit the removal of the membrane for transfer to the culture medium or be suitable for carrying out the incubation after adding the culture medium to the apparatus itself. The entire unit may be assembled and sterilized with the membrane(s) in place prior to use in the test, or the membranes may be sterilized separately by steam under pressure, or by any method that yields proper performance.

Where the article to be tested is an oil, sterilize the membrane separately, and after thorough drying, assemble the unit, using aseptic precautions.

Dissolving, diluting and rinsing fluids The following fluids are to be used for dissolving, diluting or rinsing articles under tests for sterility. They must be sterile and do not have antibacterial or antifungal properties.

FLUID A Dissolve 1 g of either *peptic digest of animal tissue* or *meat peptone* in *water* to make 1000 ml, filter or centrifuge to clarify, if necessary. Adjust to a pH of 7.1 ± 0.2, dispense into containers, and sterilize in an autoclave using a validated process.

FLUID B Prepare Fluid B by adding 1 g of *polysorbate 80* to each litre of Fluid A. Adjust to pH 7.1 ± 0.2, dispense into flasks, and sterilize in an autoclave using a validated process.

FLUID C Dissolve 5 g of either *peptic digest of animal tissue* or *meat peptone*, 3 g of *beef extract* and 10 g of *polysorbate 80* in *water* to make 1000 ml, filter or centrifuge to clarify, if necessary. Adjust to a pH of 6.9 ± 0.2, dispense into flasks, and sterilize in an autoclave using a validated process.

FLUID D Adjust, if necessary, the pH of *isopropyl myristate* to be used as Fluid D to not less than 5.5, and sterilize by filtration through a 0.22-µm membrane filter. Fluid D must also be free from antimicrobial properties.

Generally, Fluid A is used for dissolving water-soluble solids, for diluting before filtration the liquid article miscible with aqueous vehicles, and for washing the membrane(s) by filtering through the latter thereafter. If the article under test contains lecithin or oil, substitute Fluid B for Fluid A.

Fluid C is used for rinsing or washing of the filtration membrane(s), in case the article under test contains petrolatum.

Fluid D is used to dissolve or dilute ointments and oils soluble in isopropyl myristate.

Procedure Either one or two filtering units may be used, and, after filtration, the membrane half, or the whole membrane, is transferred to each of the medium used.

(A) LIQUIDS Aseptically transfer a small quantity (sufficient to moisten the membrane) of a suitable, sterile diluting fluid (Fluid A or Fluid B) onto the membrane and filter. Remove liquids from test containers of the article being examined with a sterile pipette or with a sterile syringe and needle.

For each medium to be used, transfer to a membrane not less than the quantity that is prescribed in Table 3, if necessary after diluting to about 100 ml with a suitable sterile diluting fluid. Filter immediately. (If the article is a viscous liquid or suspension not adaptable to rapid filtration, aseptically add a sufficient quantity of diluting fluid to the pooled specimen to increase the flow rate.) In case the liquid being tested has antimicrobial properties, or contains a preservative, use *Fluid A*, or *Fluid B* and proceed as directed for Membrane Filtration Method under *Validation Tests for Bacteriostasis and Fungistasis*, but exclude inoculation of the final rinse with challenge organisms.

Either transfer a membrane to each of the culture media used, or transfer each medium onto a membrane in the apparatus, and seal the apparatus so that the medium remains on the membrane.

Alternatively, transfer the combined quantity of the article being examined for both media to the membrane, diluting if necessary, filtering and washing as above. Aseptically remove and cut the membrane into two approximately equal parts and transfer one of them to each medium used.

Incubate the media for not less than 14 days unless otherwise prescribed in the monograph, at 30° to 35° in the test intended to detect bacteria and at 20° to 25° in the test intended to detect fungi. In some cases, where the liquid is highly viscous and not readily filterable through one or two membranes, more than two filter assemblies may be needed. In such cases, half the number of membranes used are incubated in each medium, provided that the volumes and requirements for numbers of containers per medium are complied with.

(B) OILS AND OILY SOLUTIONS For each medium, use not less than the quantity of the article being examined, that is prescribed in Table 3, if necessary after diluting to about 100 ml with Fluid D. Oils or oily solutions of sufficiently low viscosity may be filtered, with or without dilution, through a dry membrane. Viscous oils may be diluted as necessary with Fluid D. Allow the oil to penetrate the membrane and filter, applying pressure or suction gradually. Wash the membrane by filtering through it at least two successive quantities, each of approximately 200 ml, of Fluid B, and then wash with 100 ml of Fluid A. Complete the test described under Liquids, except that the sterility test medium to be used contains 1 g of *polysorbate 80* per litre.

(C) SOLUBLE SOLIDS For each medium, dissolve not less than the quantity of the article being examined that is prescribed in Table 4 in a suitable sterile fluid such as Fluid A and carry out the test described under Liquids using a membrane or membranes appropriate to the chosen fluid.

(D) OINTMENTS AND CREAMS For each medium, dissolve not less than 100 mg from each of not less than 20 containers in at least 100 ml of Fluid D, warming if necessary, to not more than 40°. In exceptional cases it may be necessary to heat to not more than 45°. Use warm solutions for washing. Filter as rapidly as possible and complete the test as described under Oils and Oily Solutions.

If the article under test contains petrolatum, use Fluid C in place of Fluid B for washing and moisten the membrane(s) with approximately 200 µl of Fluid C before the filtration operation begins, and keep the membrane(s) covered with liquid throughout the filtration operation for maximum efficiency of the filter. Following filtration of the specimen, wash the membrane(s) with three 100-ml portions of Fluid C. Treat the test membrane(s) as directed above.

(E) DEVICES Devices that are required to contain sterile pathways may be tested for sterility by the membrane filtration technique as follows.

Aseptically pass a sufficient volume of Fluid B through each device tested so that not less than 100 ml is recovered from each device. Collect the fluids in sterile containers, and filter the entire volume collected through membrane(s) as described under Liquids.

Method II: Direct Inoculation For each medium, use the quantity of the article being examined that is prescribed in Table 2. Eliminate any antimicrobial properties as previously described for *Direct Inoculation Method* under *Validation Tests for Bacteriostasis and Fungistasis*. Transfer the article directly into the culture medium so that volume of the product is not more than 10 per cent of the volume of the medium, unless otherwise prescribed. (A larger volume may be required if antimicrobial properties are eliminated by dilution.) For those liquid articles where it is necessary to use a large volume of the article being examined, it may be preferable to use a concentrated culture medium prepared in such a way that it takes account of the subsequent dilution. In appropriate cases the concentrated medium may be added directly to the article in its container.

(A) LIQUIDS Unless otherwise directed in the individual monograph, test 20 units of the article with each medium. Sterility tests are applied to individual discrete units or to composites of such units.

For liquid articles from each unit, use not less than the volumes of article and medium specified in Table 3. If the contents are of sufficient quantity, they may be divided so that portions are added to the two specified media.

Remove liquids from test containers with a sterile pipette or with a sterile syringe and needle. Aseptically transfer the specified volume of the material from each test container to a vessel of culture medium. Mix the liquid with the medium, but do not aerate excessively. Incubate in the specified media for not less than 14 days.

Where the material being tested renders the medium turbid, so that the presence or absence of microbial growth cannot be determined readily by visual examination, transfer suitable portions of the medium to fresh vessels of the same medium between the third and seventh days after the test is started. Continue incubation of the original and of the transfer vessels for not less than 7 additional days after the transfer and for a total of not less than 14 days.

(B) OILY LIQUIDS For oily liquids use media to which have been added 1 per cent w/v of polysorbate 80 or another suitable emulsifying agent in an appropriate concentration shown not to have antimicrobial properties under the conditions of the test. Proceed as directed under Liquids.

Aerobic cultures containing oily liquids should be shaken gently each day during the incubation period.

(C) OINTMENTS AND OILS INSOLUBLE IN ISOPROPYL MYRISTATE Select 20 containers, assign them to two groups of 10 containers, and treat each group as follows. Aseptically transfer 100 mg from each of the 10 containers to a flask containing 200 ml of a sterile, aqueous vehicle capable of dispersing the test material homogeneously throughout the fluid mixture. (The choice of dispersing agent incorporated in the aqueous vehicle may differ according to the nature of the ointment or oil. Before use, test the dispersing agent to ascertain that in the concentration used it has no significant antimicrobial effects during the time interval for all transfers, using the test procedures set forth in *Direct Inoculation Method* under *Validation Tests for Bacteriostasis and Fungistasis*.) Mix 20 ml of the fluid mixture so obtained with 200 ml of medium, and proceed as directed under Liquids.

(D) INSOLUBLE SOLIDS Transfer a quantity of the product in the form of a dry solid (or prepare a suspension of the product by adding sterile diluent to the immediate container), corresponding to not less than the quantity indicated in Table 4. Transfer the material so obtained to 200 ml of *Fluid Thioglycolate Medium*, and mix. Similarly, transfer the same quantity to 200 ml of *Soybean-Casein Digest Medium*, and mix. Proceed as directed under Liquids.

(E) COTTON, GAUZE, SURGICAL DRESSINGS, AND RELATED ARTICLES From each package of cotton, rolled gauze, or gauze bandage being tested, remove with sterile instruments two or more portions of 100 to 500 mg each from the innermost part of the sample. From individually packaged single-use materials such as gauze pads, remove a single portion of 250 to 500 mg or the entire article in the case of small, i.e., 25- by 75-mm or smaller, adhesive absorbent bandages.

Aseptically transfer these portions of the article to the similar number of containers of each medium, and incubate them as described above.

(F) SUTURES Place five containers of the sutures being examined in a suitable antimicrobial solution containing *crystal violet* or another suitable dye, for not less than 3 hours. Remove with sterile forceps, and if the containers show no evidence of leakage, hold under aseptic conditions prior to testing. Open the containers aseptically, and with sterile instruments transfer sutures to separate containers of appropriate media. Incubate them as described above for not less than 14 days.

Carry out also the test for the presence of antimicrobial activity in the suture being examined and ensure the neutralization of any inhibitory effects which, for catgut and other sutures, may be due, in part, to the methods of sterilization used or to the constituents of the tubing fluid.

(G) STERILIZED DEVICES Articles can be immersed intact or disassembled. To ensure that device pathways are also in contact with the media, immerse the appropriate number of units per medium in a volume of medium sufficient to immerse the device completely, and incubate them as described above for not less than 14 days.

For catheters where the inside lumen and outside are required to be sterile, either cut them into pieces such that the medium is in contact with the entire lumen or fill the lumen with medium, and then immerse the intact unit.

For extremely large devices, immerse those portions of the device that are to come into contact with the patient in a volume of medium sufficient to achieve complete immersion of those portions.

Observation and Interpretation of Results

At intervals during the incubation period and at its conclusion, examine the contents of all of the vessels for macroscopic evidence of microbial growth, such as the development of turbidity. If no evidence of growth is found, the material tested meets the requirements of the test for sterility. If evidence of microbial growth is found, and confirmed microscopically, the material test fails to meet the requirements of the test for sterility, unless it can be demonstrated that microbial growth observed in the test was due to inadequate aseptic sampling and testing technique rather than to intrinsic contamination of the article, the test is invalid and must be repeated. If microbial growth is not observed, the material tested meets the requirements of the sterility test. If microbial growth is observed and confirmed microscopically, the material tested does not meet the requirements of the sterility test.

10.2 MICROBIAL LIMIT TESTS

pp. 476-486, 679

Change to read:

10.2 MICROBIAL LIMIT TESTS

The hazard of microbiological contamination in non-sterile pharmaceuticals has been well realized, especially in those products of vegetable, animal and mineral origins and in those which lack good manufacturing practice (GMP). This chapter, therefore, provides tests for the estimation of the number of viable aerobic micro-organisms present and for freedom from designated microbial species, both aerobic and anaerobic, in pharmaceutical products of all kinds. An alternative or automated method, however, may be substituted for the tests presented here, provided it has been properly validated as giving equivalent or better results. The alternative method is considered invalid if the results of a control test carried out on the substance being examined using the number and type of organism specified in the tests below do not indicate the presence of the added organism, which was introduced by direct inoculation of the control preparation.

In the following, the term "micro-organism" is covering bacteria and fungi only; the term "pharmaceuticals" means pharmaceutical products of any kind, from raw materials to the finished forms; the term "growth" is used to designate the presence and presumed proliferation of viable micro-organisms.

In preparing for and in applying the tests, precautions in handling the samples should be taken so as to avoid the accidental contamination of the product and the samples being examined, as well as the inadvertent suppression of the growth of any micro-organisms which should be revealed in the test.

Buffer Solution and Media

Culture media may be prepared as follows, or dehydrated culture media may be used if they have similar or comparable nutritive and selective properties for the micro-organisms to be tested for.

In preparing the media according to the formulae set forth herein, dissolve the soluble solids in the water, using heat, if necessary, to effect complete solution, and add other ingredients. Add, if necessary, a solution of hydrochloric acid or sodium hydroxide in quantities sufficient to yield the desired pH in the medium when it is ready for use. Determine the pH at 25° ± 2°.

Where agar is called for in a formula, use agar that has a moisture content of not more than 15 per cent.

Unless otherwise indicated, the buffer solution and media should be dispensed and sterilized by heating in an autoclave at 121° for not less than 15 minutes, depending on the volume to be sterilized. Store under refrigeration.

BUFFER SOLUTION

Buffered Sodium Chloride-Peptone Solution pH 7.0

Potassium Dihydrogenphosphate	3.56 g
Disodium Hydrogenphosphate Heptahydrate	10.89 g
Sodium Chloride	4.30 g

Peptone, Dried	1.0	g
Water	1000	ml

0.1 per cent w/v to 1.0 per cent w/v of *polysorbate 20* or *80* may be added.

pH after sterilization: 7.0 ± 0.1.

MEDIA

I. Fluid Casein Digest-Soy Lecithin-Polysorbate 20 Medium

Pancreatic Digest of Casein	20.0	g
Soy Lecithin	5.0	g
Polysorbate 20	40	ml
Water	960	ml

Dissolve *pancreatic digest of casein* and *soy lecithin* in 960 ml of *water*, heating in a water-bath at 48° to 50° for about 30 minutes to effect solution. Add 40 ml of *polysorbate 20*. Mix and dispense as desired.

II. Soybean-Casein Digest Agar Medium

Pancreatic Digest of Casein	15.0	g
Papaic Digest of Soybean Meal	5.0	g
Sodium Chloride	5.0	g
Agar	15.0	g
Water	1000	ml

Prepare as directed under *Buffer Solution and Media*.

pH after sterilization: 7.3 ± 0.2.

III. Fluid Soybean-Casein Digest Medium

Pancreatic Digest of Casein	17.0	g
Papaic Digest of Soybean Meal	3.0	g
Sodium Chloride	5.0	g
Dipotassium Hydrogenphosphate	2.5	g
Dextrose Monohydrate	2.5	g
Water	1000	ml

Prepare as directed under *Buffer Solution and Media*.

pH after sterilization: 7.3 ± 0.2.

IV. Mannitol-Salt Agar Medium

Pancreatic Digest of Casein	5.0	g
Papaic Digest of Animal Tissue	5.0	g
Beef Extract	1.0	g
Mannitol	10.0	g
Sodium Chloride	75.0	g
Agar	15.0	g
Phenol Red	25.0	g
Water	1000	ml

Mix, then heat with frequent agitation, and boil for 1 minute to effect solution.

pH after sterilization: 7.4 ± 0.2.

V. Baird-Parker Agar Medium

Pancreatic Digest of Casein	10.0	g
Beef Extract	5.0	g
Yeast Extract	1.0	g
Lithium Chloride	5.0	g
Agar	20.0	g
Glycine	12.0	g
Sodium Pyruvate	10.0	g
Water	950	ml

Heat with frequent agitation, and boil for 1 minute. Sterilize, cool to between 45° and 50°, and add 10 ml of a sterile 1 per cent w/v solution of *potassium tellurate(IV)* and 50 ml of egg-yolk emulsion. Mix intimately but gently, and pour into plates.

pH after sterilization: 6.8 ± 0.2.

Preparation of the egg-yolk emulsion: Disinfect the surface of whole shell eggs, aseptically crack the eggs, and separate out intact yolks into a sterile graduated cylinder. Add saline TS to obtain a 3 to 7 ratio of egg-yolk to *saline*. Add to a sterile blender cup, and mix at high speed for 5 seconds.

VI. Vogel-Johnson Agar Medium

Pancreatic Digest of Casein	10.0	g
Yeast Extract	5.0	g
Mannitol	10.0	g
Dipotassium Hydrogenphosphate	5.0	g
Lithium Chloride	5.0	g
Glycine	10.0	g
Agar	16.0	g
Phenol Red	25.0	mg
Water	1000	ml

Boil the solution of solids for 1 minute. Sterilize, cool to between 45° and 50°, and add 20 ml of a sterile 1 per cent w/v solution of *potassium tellurate(IV)*.

pH after sterilization: 7.2 ± 0.2.

VII. Cetrimide Agar Medium

Pancreatic Digest of Gelatin	20.0	g
Magnesium Chloride	1.4	g
Potassium Sulfate	10.0	g
Agar	13.6	g
Cetrimide	0.3	g
Glycerol	10.0	ml
Water	1000	ml

Dissolve all solid components in *water*, and add *glycerol*. Heat, with frequent agitation, and boil for 1 minute to effect solution.

pH after sterilization: 7.2 ± 0.2.

VIII. Pseudomonas Agar Medium for Detection of Fluorescin

Pancreatic Digest of Casein	10.0	g
Peptic Digest of Animal Tissue	10.0	g
Dipotassium Hydrogenphosphate	1.5	g
Magnesium Sulfate	1.5	g
Agar	15.0	g
Glycerol	10.0	ml
Water	1000	ml

Dissolve the solid components in *water* before adding *glycerol*. Heat, with frequent agitation, and boil for 1 minute to effect solution.

pH after sterilization: 7.2 ± 0.2.

IX. Pseudomonas Agar Medium for Detection of Pyocyanin

Pancreatic Digest of Gelatin	20.0	g
Magnesium Chloride	3.0	g
Potassium Sulfate	10.0	g
Agar	15.0	g
Glycerol	10.0	ml
Water	1000	ml

Dissolve the solid components in *water* before adding *glycerol*. Heat, with frequent agitation, and boil for 1 minute to effect solution.

pH after sterilization: 7.2 ± 0.2.

X. Fluid Lactose Medium

Beef Extract	3.0	g
Pancreatic Digest of Gelatin	5.0	g
Lactose	5.0	g
Water	1000	ml

Cool as quickly as possible after sterilization.

pH after sterilization: 6.9 ± 0.2.

XI. Rappaport-Vassiliadis Broth

Soya Peptone	4.5	g
Sodium Chloride	8.0	g
Dipotassium Phosphate	0.4	g
Potassium Dihydrogenphosphate	0.6	g
Magnesium Chloride	29.0	g
Malachite Green	36.0	mg
Water	1000	ml

Mix and heat to effect solution.

pH after sterilization: 5.2 ± 0.2.

XII. Fluid Tetrathionate Medium

Pancreatic Digest of Casein	2.5	g
Peptic Digest of Animal Tissue	2.5	g
Bile Salts	1.0	g
Calcium Carbonate	10.0	g
Sodium Thiosulfate	30.0	g
Water	1000	ml

Heat the solution of solids to boiling. On the day of use, add a solution prepared by dissolving 5 g of *potassium iodide* and 6 g of *iodine* in 20 ml of *water*. Then add 10 ml of a 0.1 per cent w/v solution of *brilliant green*, and mix. Do not heat the medium after adding the brilliant green solution.

pH after sterilization: 7.0 ± 0.2.

XIII. Brilliant Green Agar Medium

Yeast Extract	3.0	g
Peptic Digest of Animal Tissue	5.0	g
Pancreatic Digest of Casein	5.0	g
Lactose	10.0	g
Sodium Chloride	5.0	g
Sucrose	10.0	g
Phenol Red	80.0	mg
Agar	20.0	g
Brilliant Green	12.5	mg
Water	1000	ml

Boil the solution of solids for 1 minute. Sterilize just prior to use. Melt the medium, pour into Petri dishes, and allow to cool.

pH after sterilization: 6.9 ± 0.2.

XIV. Xylose-Lysine-Desoxycholate Agar Medium

Xylose	3.5	g
L-Lysine	5.0	g
Lactose	7.5	g
Sucrose	7.5	g
Sodium Chloride	5.0	g
Yeast Extract	3.0	g
Agar	13.5	g
Sodium Desoxycholate	2.5	g
Sodium Thiosulfate	6.8	g
Ammonium Iron(III) Citrate	0.8	g
Phenol Red	80.0	mg
Water	1000	ml

Heat the mixture of solids and *water*, with swirling, just to the boiling point. *Do not overheat or sterilize*. Transfer at once to a water-bath maintained at about 50°, and pour into plates as soon as the medium has cooled.

Final pH: 7.4 ± 0.2.

XV. Bismuth Sulfite Agar Medium

Beef Extract	5.0	g
Pancreatic Digest of Casein	5.0	g
Peptic Digest of Animal Tissue	5.0	g
Dextrose Monohydrate	5.0	g
Disodium Hydrogenphosphate	4.0	g

Heptahydrate		
Iron(II) Sulfate	0.3	g
Bismuth Sulfite Indicator	8.0	g
Agar	20.0	g
Brilliant Green	25.0	mg
Water	1000	ml

Heat the mixture of solids and *water*, with swirling, just to the boiling point. *Do not overheat or sterilize.* Transfer at once to a water-bath maintained at about 50°, and pour into plates as soon as the medium has cooled.

Final pH: 7.6 ± 0.2.

XVI. Triple Sugar-Iron-Agar Medium

Pancreatic Digest of Casein	10.0	g
Pancreatic Digest of Animal Tissue	10.0	g
Lactose	10.0	g
Sucrose	10.0	g
Dextrose Monohydrate	1.0	g
Ammonium Iron(II) Sulfate	0.2	g
Sodium Chloride	5.0	g
Sodium Thiosulfate	0.2	g
Agar	13.0	g
Phenol Red	25.0	mg
Water	1000	ml

Prepare as directed under *Buffer Solution and Media.*

pH after sterilization: 7.3 ± 0.2.

XVII. Fluid Enterobacteria Enrichment Medium

Pancreatic Digest of Gelatin	10.0	g
Dextrose Monohydrate	5.0	g
Dehydrated Ox Bile	20.0	g
Potassium Dihydrogenphosphate	3.0	g
Disodium Hydrogenphosphate Dihydrate	8.0	g
Brilliant Green	15.0	mg
Water	1000	ml

Mix and heat at 100° for 30 minutes to sterilize and cool immediately. *Do not autoclave.*

Final pH: 7.2 ± 0.2.

XVIII. Crystal Violet-Neutral Red-Bile-Dextrose Agar Medium

Yeast Extract	3.0	g
Pancreatic Digest of Gelatin	7.0	g
Bile Salts Mixture	1.5	g
Lactose	10.0	g
Sodium Chloride	5.0	g
Dextrose Monohydrate	10.0	g
Agar	15.0	g
Neutral Red	30.0	mg
Crystal Violet	2.0	mg
Water	1000	ml

Mix and heat to boiling. *Do not overheat or sterilize.* Transfer at once to a water-bath maintained at about 50°, and pour into plates as soon as the medium has cooled.

Final pH: 7.4 ± 0.2.

XIX. MacConkey Agar Medium

Pancreatic Digest of Gelatin	17.0	g
Pancreatic Digest of Casein	1.5	g
Peptic Digest of Animal Tissue	1.5	g
Lactose	10.0	g
Bile Salts Mixture	1.5	g
Sodium Chloride	5.0	g
Agar	13.5	g
Neutral Red	30.0	mg
Crystal Violet	1.0	mg
Water	1000	ml

Boil the mixture of solids and *water* for 1 minute to effect solution.

pH after sterilization: 7.1 ± 0.2.

XX. Levine Eosin-Methylene Blue Agar Medium

Pancreatic Digest of Gelatin	10.0	g
Dipotassium Hydrogenphosphate	2.0	g
Agar	15.0	g
Lactose	10.0	g
Eosin Y	0.4	g
Methylene Blue	65.0	mg
Water	1000	ml

Dissolve *pancreatic digest of gelatin, dipotassium hydrogenphosphate* and *agar* in *water*, with warming, and allow to cool. Just prior to use, liquefy the gelled agar solution, add the remaining ingredients, as solutions, in the following amounts, and mix: for each 100 ml of the liquefied agar solution—5 ml of a 20 per cent w/v solution of *lactose*, 2 ml of a 2 per cent w/v solution of *eosin Y*, and 2 ml of a 0.33 per cent w/v solution of *methylene blue*. The finished medium may not be clear.

pH after sterilization: 7.1 ± 0.2.

XXI. Sabouraud Dextrose Agar Medium

Dextrose Monohydrate	40.0	g
Mixture of Equal Parts of *Peptic Digest of Animal Tissue* and *Pancreatic Digest of Casein*	10.0	g
Agar	15.0	g

Water	1000	ml

Mix and boil to effect solution.

pH after sterilization: 5.6 ± 0.2.

XXIa. Sabouraud Dextrose Agar Medium with Antibiotics

Dextrose Monohydrate	40.0	g
Mixture of Equal Parts of *Peptic Digest of Animal Tissue and Pancreatic Digest of Casein*	10.0	g
Agar	15.0	g
Water	1000	ml

Mix and boil to effect solution. Immediately before use, add 0.10 g of *benzylpenicillin sodium* and 0.10 g of *tetracycline* per litre of medium as sterile solutions or alternatively, add 50 mg of *chloramphenicol* per litre of medium before sterilization.

pH after sterilization: 5.6 ± 0.2.

XXII. Potato Dextrose Agar Medium

Cook 300 g of peeled and diced potatoes in 500 ml of water prepared by distillation, filter through cheesecloth, add water prepared by distillation to make 1000 ml, and add the following:

Agar	15.0	g
Dextrose Monohydrate	20.0	g

Dissolve by heating and sterilize.

pH after sterilization: 5.6 ± 0.2.

For use, just prior to pouring the plates, adjust the melted and cooled to 45° medium with a sterile 10 per cent w/v solution of *tartaric acid* to a pH of 3.5 ± 0.1. Do not reheat the pH 3.5 medium.

XXIII. Cooked-Meat Medium

Part I

Beef Heart (fat free)	454.0	g
1 M *Sodium Hydroxide*	25	ml
Water	1000	ml

Mix ground beef heart with 1000 ml of *water* and add 25 ml of 1 M *sodium hydroxide*. Heat to boiling and simmer for 20 minutes with frequent stirring. Cool and check the pH, which should be about 7.2; adjust it if necessary. Filter through several layers of gauze; squeeze out the excess of liquid. Spread the meat particles to partially dry and place in suitable vessels.

Part II

Filter the fluid obtained from part I through three pieces of coarse filter paper to clarify. Then filter through one piece of finer filter paper (Whatman No.1 or equivalent is suitable). Dissolve the following ingredients in the filtrate:

Peptic Digest of Animal Tissue	20.0	g
Dextrose Monohydrate	2.0	g
Sodium Chloride	5.0	g

and adjust the volume to 1000 ml with *water*. Add the fluid to the vessels, using about four to five parts of the fluid to one part of the meat.

pH after sterilization: 7.2 ± 0.1.

XXIV. 5 Per Cent Defibrinated Sheep Blood Agar Medium (Blood Agar Medium)

Heat Soybean-Casein Digest Agar Medium (Medium II) and cool to 45° to 50° in a water-bath. Add sufficient amount of defibrinated sheep blood to make 5 per cent and mix.

XXV. Fluid Thioglycolate Medium (Fluid Mercaptoacetate Medium)

L-Cystine	0.5	g
Sodium Chloride	2.5	g
Dextrose Monohydrate	5.5	g
Agar, granulated	0.75	g
Yeast Extract	5.0	g
Pancreatic Digest of Casein	15.0	g
Sodium Thioglycolate (or *Thioglycolic Acid* 0.3 ml)	0.5	g
Resazurin Sodium Solution (0.01 per cent w/v, freshly prepared)	1.0	ml
Water	1000	ml

Prepare as directed under *Buffer Solution and Media*.

pH after sterilization: 7.1 ± 0.2.

XXVI. Iron-Milk Medium, Modified

Fresh Whole Milk	1000	ml
Iron(III) Sulfate	1.0	g
Water	50	ml

Dissolve *iron(III) sulfate* in 50 ml of *water* and add slowly to 1000 ml of fresh whole milk while mixing with a magnetic stirrer. Dispense 11 ml of the Iron-Milk Medium into a 16-mm × 150-mm culture tube and sterilize. Prepare fresh medium for each use.

XXVII. Egg-Yolk Agar Medium

Peptic Digest of Animal Tissue	40.0	g
Disodium Hydrogenphosphate Dihydrate	5.0	g
Potassium Dihydrogenphosphate	1.0	g
Sodium Chloride	2.0	g
Magnesium Sulfate	0.1	g
Dextrose Monohydrate	2.0	g

Agar	25.0	g
Water	1000	ml

Heat with frequent agitation, and boil for 1 minute. Sterilize in the autoclave for 20 minutes at 121° and cool to 50°. Add 100 ml of egg-yolk emulsion. Mix thoroughly and pour into plates. (Prepare the egg-yolk emulsion as described under Baird-Parker Agar Medium (Medium V) except using a ratio of egg-yolk to *saline TS* of 1:1.)

pH after sterilization: 7.6 ± 0.2.

XXVIII. MacConkey Broth

Pancreatic Digest of Gelatin	20.0	g
Lactose	10.0	g
Dehydrated ox bile	5.0	g
Bromocresol Purple	10	mg
Water	1000	ml

Prepare as directed under *Buffer Solution and Media*.

pH after sterilization: 7.3 ± 0.2.

XXIX. Reinforced Medium for Clostridia

Beef Extract	10.0	g
Peptone	10.0	g
Yeast extract	3.0	g
Soluble Starch	1.0	g
Dextrose Monohydrate	5.0	g
Cysteine Hydrochloride	0.5	g
Sodium Chloride	5.0	g
Sodium Acetate	3.0	g
Agar	0.5	g
Water	1000	ml

Hydrate the agar, and dissolve by heating to boiling with continuous stirring.

pH after sterilization: 6.8 ± 0.2.

XXX. Columbia Agar

Pancreatic Digest of Casein	10.0	g
Peptic Digest of Animal Tissue	5.0	g
Heart Pancreatic Digest	3.0	g
Yeast Extract	5.0	g
Maize Starch	1.0	g
Sodium Chloride	5.0	g
Agar, according to gelling power	10.0 to 15.0	g
Water	1000	ml

Hydrate the agar, and dissolve by heating to boiling with continuous stirring. Sterilize, cool to between 45° and 50° and add, when necessary, gentamicin sulfate corresponding to 20 mg of gentamicin base. Pour into Petri dishes.

pH after sterilization: 7.3 ± 0.2.

Test for Nutritive and Selective Properties of the Media and Validity of the Test for Specified Micro-organisms

Test each medium for its nutritive and selective properties and for the validity of the test for specified micro-organisms, as well as for the antimicrobial agent(s) in the material being examined, as follows.

Grow the following test strains (Table 1) separately in tubes containing the indicated media at 30° to 35° for 18 to 24 hours for aerobic bacteria and at 35° to 37° for up to 4 days for anaerobic bacteria.

Dilute portions of each of the cultures using Buffered Sodium Chloride-Peptone Solution pH 7.0 to make test suspensions. Use about 100 viable micro-organisms per ml of each strain as an inoculate in tests for *Staphylococcus aureus*,

Table 1 Test Strains for Specified Micro-organisms

Micro-organism	Strain*	Medium
Staphylococcus aureus	ATCC 6538 (NCIMB 9518)	Fluid Medium III
Pseudomonas aeruginosa	ATCC 9027 (NCIMB 8626)	Fluid Medium III
Escherichia coli	ATCC 8739 (NCIMB 8545)	Fluid Medium III
*Salmonella abony***	NCTC 6017	Fluid Medium III
Bacillus anthracis	34F2†	Fluid Medium III
Clostridium sporogenes	ATCC 19404 (NCTC 532)	Medium XXIII
	ATCC 11437 (DMST 15536)	Medium XXIII

* Other available nonpathogenic strains may be used.
** Other available nonpathogenic salmonellae may also be used.
† Non-virulent strain available from Veterinary Biologics Division, Department of Livestock Development, Ministry of Agriculture and Cooperatives.

Pseudomonas aeruginosa, *Escherichia coli*, *Salmonella* spp., *Bacillus anthracis*, and *Clostridium sporogenes* in the presence and absence of the sample to be examined, if necessary. Each suspension of micro-organism can be used to perform separately as a control of the counting method. When testing the method described under *Total Viable Aerobic Microbial Count* and *Test for Specified Micro-organisms*, a positive result for the respective micro-organism should be obtained. The tests are invalid if the results do not indicate the presence of the added micro-organisms (see also *Validation of the Counting Method*). Failure of the micro-organism(s) to grow in the absence of the sample to be examined invalidates the nutritive and the selective values of the medium. On the other hand, if no growth is observed in the presence of the sample, it indicates the presence of inhibitory substance(s) in the sample taken and necessitates a modification of the procedure by any of the following methods.

a. Dilution—an increase in volume of diluent or culture medium, the quantity of the test material remaining the same.

b. Neutralization—the incorporation of a sufficient quantity of suitable inactivating agent(s) in the diluent or in the culture medium to neutralize the inhibitory substance(s) present in the sample; the examples of these inhibitory substance are: 0.5 per cent of soy lecithin and 4.0 per cent of polysorbate 20. Alternatively, Fluid Casein Digest-Soy Lecithin-Polysorbate 20 Medium (Medium I) may be used to demonstrate neutralization of preservatives or other antimicrobial agents in the test material.

c. An appropriate combination of modifications (a) and (b) so as to permit growth of the inocula.

d. Membrane filtration, with subsequent washing out of the inhibitory substance(s), if the sample is soluble.

If, in spite of the incorporation of suitable inactivating agents and a substantial increase in the volume of diluent, it is still not possible to recover the viable cultures described above and where the sample is not suitable for employment of membrane filtration, it can be assumed that the failure to isolate the inoculated organism is due to the bactericidal activity of the product. This information serves to indicate that the sample is not likely to be contaminated with the given species of micro-organism. Monitoring should be continued in order to establish the spectrum of inhibition and bactericidal activity of the sample.

Sampling

With due precautions referred to above in the handling of the sample to be examined, select at random the contents of several containers (at least 3) or several portions from the bulk material, mix, and render them, as far as possible, homogeneous. Unless otherwise prescribed, use not less than 10 g or 10 ml of the homogeneous sample, accurately weighed or measured.

Preparation of Sample

In order to obtain a solution or suspension in a form suitable for the test to be carried out, prepare the sample to be tested by treatment that is appropriate to its physical characteristics and that does not alter the number and kind of micro-organisms originally present.

WATER-SOLUBLE SAMPLES Dilute or dissolve 10.0 g or 10.0 ml of the sample to be examined in Buffered Sodium Chloride-Peptone Solution pH 7.0, and make up to 100.0 ml with the same solution.

NON-FATTY SAMPLES INSOLUBLE IN WATER Suspend 10.0 g, if necessary after pulverization, or 10.0 ml of the sample in Buffered Sodium Chloride–Peptone Solution pH 7.0 and dilute to 100.0 ml with the same solution. Some products may necessitate the use of large volumes. If necessary, homogenize the suspension mechanically. A suitable surface-active agent such as 0.1 per cent w/v *polysorbate 80* may be added to assist the suspension. Adjust, if necessary, the pH of the suspension to about 7.

FATTY SAMPLES Homogenize 10.0 g or 10.0 ml of the sample with 5 g of *polysorbate 20* or *polysorbate 80*, heated to not more than 40° (or, if necessary, to not more than 45° for the shortest possible time for some samples). Mix carefully while maintaining the temperature in a water-bath or in an oven. Add 85 ml of Buffered Sodium Chloride-Peptone Solution pH 7.0, preheated to not more than 40°, if necessary. Maintain this temperature for the shortest time, not more than 30 minutes, in the water-bath, with frequent agitation, to emulsify. If necessary, adjust the pH of the emulsion to about 7.

FLUID SAMPLE IN AEROSOL FORM Chill the container(s) in an alcohol-dry ice mixture for approximately 1 hour, cut open the container(s), and allow to reach room temperature, permitting the propellant to escape, or warming to drive off the propellant if feasible. Transfer 10.0 g or 10.0 ml of the remaining

portions to the culture medium. Where 10.0 g or 10.0 ml of the remaining portion, whichever is applicable, cannot be obtained from 10 containers in aerosol form, transfer the entire contents from 10 chilled containers to the culture medium, permitting the propellant to escape, and proceed the test on the residues.

TRANSDERMAL PATCHES Remove the protective cover sheets of ten patches of the product being examined by using sterile forceps and place them, the adhesive side upwards, on sterile glass or plastic trays. Cover the adhesive surface with sterile gauze, if necessary, and transfer the ten patches to a minimum volume of 500 ml of Buffered Sodium Chloride-Peptone Solution pH 7.0 containing suitable inactivators such as *polysorbate 80* and/or lecithin. Shake vigorously the preparation for at least 30 minutes (preparation A). Prepare another ten patches in the same way, place them in a minimum volume of 500 ml of Fluid Lactose Medium (Medium X) and shake vigorously for at least 30 minutes (preparation B).

Total Viable Aerobic Microbial Count

Unless otherwise prescribed in the particular monograph, the following methods should be used:

For soluble samples: plate method or membrane filtration method;

sufficiently soluble or translucent samples: plate method or multiple-tube method;

others: multiple-tube method.

Before carrying out the test for Total Viable Aerobic Microbial Count, perform the test for absence of inhibitory (antimicrobial) properties of the sample as described under Test for Nutritive and Selective Properties of the Media and Validity of the Test for Specified Micro-organisms.

Add the sample to the medium not more than 1 hour after preparing the appropriate dilutions for inoculation. For a viscous sample that cannot be pipetted at the initial 1:10 dilution, dilute the sample until a suspension is obtained, i.e., 1:50 or 1:100, etc., that can be pipetted.

PLATE METHOD

For bacteria Dilute further, if necessary, the prepared sample so that 1 ml will be expected to yield between 30 and 300 colonies. Pipette 1 ml of the final dilution onto each of two sterile Petri dishes, 9 to 10 cm in diameter. Promptly add to each dish 15 to 20 ml of Soybean-Casein Digest Agar Medium (Medium II) that previously has been melted and cooled to approximately 45°. Cover the Petri dishes, mix the sample with the agar by tilting or rotating the dishes, and allow the contents to solidify at room temperature. Alternatively, evenly spread (e.g. with the aid of a glass rod and a turntable) 0.1 ml of prepared sample over the surfaces of solidified and previously dried Soybean-Casein Digest Agar Medium (for example, in a laminar air flow bench or in an incubator) in two Petri dishes of the same diameter. Invert the Petri dishes, and incubate at 30° to 35° for 5 days unless a reliable count is obtained in a shorter time. Following incubation, examine the plates for growth, count the number of colonies, and express the average for the two plates in terms of the number of micro-organisms per g or per ml of the sample. If no microbial colonies are recovered from the dishes representing the initial 1:10 dilution of the sample, express the results as "less than 10 micro-organisms per g or per ml of the sample".

For fungi Dilute further, if necessary, the prepared sample so that 1 ml will be expected to yield not more than 100 colonies. Follow the same procedure as for bacteria except using Sabouraud Dextrose Agar Medium with Antibiotics (Medium XXIa) or Potato Dextrose Agar Medium (Medium XXII) and incubating at 20° to 25° for 5 days.

MEMBRANE-FILTRATION METHOD

Use membrane filters having a nominal pore size not greater than 0.45 µm and whose effectiveness to retain micro-organisms has been established. Cellulose nitrate filters, for example, are used for aqueous, oily and weakly alcoholic solutions, and cellulose acetate filters for strongly alcoholic solutions. Preferably, membranes of about 50 mm in diameter are recommended. The filtration apparatus and membrane must be sterilized by appropriate means. The apparatus should be so designed that the solution to be examined can be introduced and filtered under aseptic conditions, and it should permit the removal of the membrane for transfer to the culture medium. If necessary, dilute the prepared sample so that a colony of 10 to 100 may be expected. Transfer 10 ml to each of two membrane filters and filter immediately. Wash each membrane by passing through the filter at least three 100-ml portions of a suitable liquid such as Buffered Sodium Chloride-Peptone Solution pH 7.0. For fatty substances, this liquid may contain a suitable

surface-active agent, such as polysorbate 20 or polysorbate 80. Transfer one of the membrane filters, intended primarily for the enumeration of bacteria, to the surface of a plate of Soybean-Casein Digest Agar Medium, and the other, intended primarily for the enumeration of fungi, to the surface of a plate of Sabouraud Dextrose Agar Medium with Antibiotics. Incubate the plate of Soybean-Casein Digest Agar Medium at 30° to 35° for 3 days, and the plate of Sabouraud Dextrose Agar Medium with Antibiotics at 20° to 25° for 5 days. Count the number of colonies which develop. Calculate the number of micro-organisms per g or per ml of the sample to be examined, if necessary, counting bacteria and fungi separately.

When examining transdermal patches, filter 50 ml of preparation A as described under *Preparation of Sample*, separately through each of two sterile filter membranes. Place one membrane to Soybean-Casein Digest Agar Medium for total aerobic microbial count, the other membrane to Sabouraud Dextrose Agar Medium with Antibiotics for the count of fungi.

MULTIPLE-TUBE METHOD (Most Probable Number: MPN)

Prepare three dilutions of the sample, viz 1:10, 1:100 and 1:1000, and a series of 12 tubes each containing 9 ml of sterile Fluid Soybean-Casein Digest Medium (Medium III). To each of the first three tubes add 1 ml of the 1:10 dilution of the sample. To each of the next three tubes add 1 ml of the 1:100 dilution, and to each of the next three tubes add 1 ml of the 1:1000 dilution. To the last three tubes add 1 ml of the diluent. Incubate the tubes at 30° to 35° for 5 days. Following the incubation period, examine the tubes for growth. The last three tubes should show no growth; otherwise, the test is invalid. If the reading of the results is difficult or uncertain owing to the nature of the sample examined, subculture on a liquid or solid medium and read the results after a further period of incubation. Determine the most probable number of bacteria per g or per ml of the sample from Table 2.

The Most Probable Number method is reserved for bacterial counts when no other method is available. The choice of a method may be based on factors such as the nature of the product and the expected number of micro-organisms. Any method which is chosen must be properly validated.

VALIDATION OF THE COUNTING METHOD

When testing the membrane-filtration method or the plate count method, a count of any of the test organisms differing by not more than 20 per cent from the calculated value for the inoculum is to be obtained. When testing the multiple-tube method the calculated value from the inoculum is to be within the 95 per cent confidence limits of the results obtained (Table 2).

Interpretation of the Results

The bacterial count will be considered to be equal to the average number of colony-forming units found on Soybean-Casein Digest Agar Medium. The fungal count will be considered to be equal to the average number of colony-forming units on Sabouraud Dextrose Agar Medium with Antibiotics. The total viable aerobic microbial count is the sum of the bacterial count and the fungal count as described above. If there is evidence that the same types of micro-organisms grow on both media, this may be corrected. If the count is carried out by the Most Probable Number method, the calculated value is the bacterial count.

The limits prescribed in the "Limits for Microbial Contamination" (Appendix 10.5) are the maximum acceptable limits.

Test for Specified Micro-organisms

Before carrying out the following tests, perform the tests for absence of inhibitory (antimicrobial) properties of the sample as described under Test for Nutritive and Selective Properties of the Media and Validity of the Test for Specified Micro-organisms.

ENTEROBACTERIA

Detection of bacteria To 10 g (or 10 ml) of the sample being examined, add Fluid Lactose Medium (Medium X) to make 100 ml, homogenize and incubate at 35° to 37° for about 2 (but not more than 5) hours, to revivify the bacteria. Shake the container, transfer the quantity of the contents (homogenate A) corresponding to 1 g (or 1 ml) of the sample to 100 ml of Fluid Enterobacteria Enrichment Medium (Medium XVII) and incubate for 18 to 48 hours at 35° to 37°. Subculture on plates of Crystal Violet Neutral Red-Bile-Dextrose Agar Medium (Medium XVIII). Incubate at 35° to 37° for 18 to 24 hours. The sample passes the test if there is no growth of colonies of Gram-negative bacteria on any plate.

Table 2 Most Probable Number (MPN) Values of Bacteria

Three tubes at each level of dilution							
Number of positive tubes			MPN per g or ml	Category*		95 per cent Confidence Limits	
1:10 0.1 g or 0.1 ml	1:100 0.01 g or 0.01 ml	1:1000 0.001 g or 0.001ml		A	B		
0	0	0	< 3			-	-
0	1	0	3		x	< 1	17
1	0	0	3	x		1	21
1	0	1	7		x	2	27
1	1	0	7	x		2	28
1	2	0	11		x	4	35
2	0	0	9	x		2	38
2	0	1	14		x	5	48
2	1	0	15	x		5	50
2	1	1	20		x	8	61
2	2	0	21	x		8	63
3	0	0	23	x		7	129
3	0	1	38	x		10	180
3	1	0	43	x		20	210
3	1	1	75	x		20	280
3	2	0	93	x		30	390
3	2	1	150	x		50	510
3	2	2	210		x	80	640
3	3	0	240	x		100	1400
3	3	1	460	x		200	2400
3	3	2	1100	x		300	4800
3	3	3	> 1100			-	-

*Category A: Normal results, obtained in 95 per cent of the cases.
Category B: Less likely results, obtained in only 4 per cent of cases. These are not to be used for important decisions. Results that are even less likely than those of category B are not mentioned and are always unacceptable.

Quantitative evaluation Inoculate suitable quantities of Fluid Enterobacteria Enrichment Medium with homogenate A, prepared as above, or dilutions of it, containing respectively 0.1 g (or 0.1 ml), 0.01 g (or 0.01 ml), 0.001 g (or 0.001 ml) and 0.0001 g (or 0.0001 ml) of the sample to be examined. Incubate at 35° to 37° for 24 to 48 hours. Subculture each of the cultures on a plate of Crystal Violet Neutral Red-Bile-Dextrose Agar Medium to obtain selective isolation. Incubate at 35° to 37° for 18 to 24 hours. Growth of well-developed colonies, generally red or reddish, of Gram-negative bacteria constitutes a positive result. Note the smallest quantity of the sample which gives a positive result and the largest quantity that gives a negative result. Determine from Table 3 the probable number of bacteria.

When testing transdermal patches, filter 50 ml of preparation B as described under *Preparation of Sample* through a sterile filter membrane. Place the membrane in 100 ml of Fluid Enterobacteria Enrichment Medium and incubate for 18 to 24 hours at 35° to 37°. Subculture on plates of Crystal Violet Neutral Red-Bile-Dextrose Agar Medium for detection of Enterobacteria and other Gram-negative bacteria.

SALMONELLA SPECIES

To about 10 g (or 10 ml) of the sample being examined, add 100 ml of Fluid Soybean-Casein Digest Medium, mix and incubate at 35° to 37° for 18 to 24 hours. Transfer 1 ml of the enrichment culture to 10 ml of Rappaport Vassiliadis Broth (Medium XI) and Fluid Tetrathionate Medium (Medium XII), respectively, mix and incubate at 35° to 37° for 18 to 24 hours. Subculture on plates of Brilliant Green Agar Medium (Medium XIII), Xylose-Lysine-Desoxycholate Agar Medium (Medium XIV), and

Table 3 Probable Number of Bacteria

Results for Each Quantity of Sample				Probable Number of Bacteria per g or per ml of Sample
0.1 g (or 0.1 ml)	0.01 g (or 0.01 ml)	0.001 g (or 0.001 ml)	0.0001 g (or 0.0001 ml)	
+	+	+	+	More than 10^4
+	+	+	–	Less than 10^4 and more than 10^3
+	+	–	–	Less than 10^3 and more than 10^2
+	–	–	–	Less than 10^2 and more than 10
–	–	–	–	Less than 10

Table 4 Morphology Characteristics of *Salmonella* Species on Selective Agar Media

Selective Medium	Characteristic Colonial Morphology
Brilliant Green Agar Medium	Small, transparent, colourless or pink to white opaque (frequently surrounded by pink to red zone)
Xylose-Lysine-Desoxycholate Agar Medium	Red, with or without black centres
Bismuth Sulfite Agar Medium	Black or green

Table 5 Morphology Characteristics of *Escherichia coli* on MacConkey Agar Medium

Gram Stain	Characteristic Colonial Morphology
Negative rods (cocco-bacilli)	Brick-red; may have surrounding zone of Precipitated bile

Bismuth Sulfite Agar Medium (Medium XV). Cover and invert the dishes, and incubate. Upon examination, if none of the colonies conforms to the description given in Table 4, the sample meets the requirements of the test for absence of the genus Salmonella.

If colonies of Gram-negative rods matching the description in Table 4 are found, proceed with further identification by transferring representative suspect colonies individually, by means of an inoculating wire, to a butt-slant tube of Triple Sugar-Iron Agar Medium (Medium XVI) by first streaking the surface of the slant and then stabbing the wire well beneath the surface, and incubate. If examination discloses no evidence of tubes having alkaline (red) slants and acid (yellow) butts (with or without concomitant blackening of the butt from hydrogen sulfide production), the sample meets the requirements of the test for absence of the genus Salmonella. The presence of Salmonella may be confirmed by other suitable cultural or biochemical and serological tests, if necessary.

ESCHERICHIA COLI

Prepare the product being examined as described under *Preparation of Sample* and use 10 ml or the portion corresponding to 1 g or 1 ml to inoculate 100 ml of Fluid Soybean-Casein Digest Medium, mix and incubate at 35° to 37° for 18 to 48 hours. Shake the container, transfer 1 ml to 100 ml of MacConkey Broth (Medium XXVIII) and incubate at 43° to 45° for 18 to 24 hours. Subculture on plates of MacConkey Agar Medium (Medium XIX) and incubate at 35° to 37° for 18 to 72 hours. Upon examination, if none of the colonies conforms to the description given in Table 5, the sample meets the requirements of the test for absence of *Escherichia coli*.

If colonies matching the description in Table 5 are found, proceed with further identification by

transferring the suspect colonies individually, making subculture the suspect colonies individually on plate of Levine Eosin-Methylene Blue Agar Medium (Medium XX), and incubate at 35° to 37° for 18 to 24 hours. Upon examination, if none of the colonies exhibits both a characteristic metallic sheen under reflected light and a blue-black appearance under transmitted light, the sample meets the requirements of the test for absence of *Escherichia coli*. The presence of *Escherichia coli* may be confirmed by further suitable cultural or biochemical and serological tests.

STAPHYLOCOCCUS AUREUS AND PSEUDOMONAS AERUGINOSA

Prepare the product being examined as described under *Preparation of Sample* and use 10 ml or the portion corresponding to 1 g or 1 ml to inoculate 100 ml of Fluid Soybean-Casein Digest Medium. Mix and incubate at 35° to 37° for 18 to 48 hours. Examine the medium for growth, and if growth is present, use an inoculating loop to streak a portion of the medium on the surface of Mannitol-Salt Agar Medium (Medium IV), or Baird-Parker Agar Medium (Medium V), or Vogel-Johnson Agar Medium (Medium VI) and of Cetrimide Agar Medium (Medium VII), and incubate at 35° to 37° for 18 to 72 hours. If upon examination, none of the plates contains colonies having the characteristics listed in Tables 6 and 7 for the media used, the test sample meets the requirements for the absence of *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

Coagulase test (for *Staphylococcus aureus*) With the aid of an inoculating loop, transfer representative suspect colonies from the agar surfaces of the Mannitol-Salt Agar Medium (or Baird-Parker Agar Medium, or Vogel-Johnson Agar Medium) to individual tubes, each containing 0.5 ml of mammalian, preferably rabbit or horse, plasma with or without suitable additives. Incubate in a water-bath at 37°, examining the tubes at 3 hours and subsequently at suitable intervals up to 24 hours. Test positive and negative controls simultaneously with the unknown samples. If no coagulation in any degree is observed, the sample meets the requirements of the test for absence of *Staphylococcus aureus*.

Oxidase and pigment tests (for *Pseudomonas aeruginosa*) With the aid of an inoculating loop, streak representative suspect colonies from the agar surfaces of Cetrimide Agar Medium on the agar surface of Pseudomonas Agar Medium for Detection of Fluorescin (Medium VIII) and Pseudomonas Agar Medium for Detection of Pyocyanin (Medium IX)

Table 6 Morphology Characteristics of *Staphylococcus aureus* on Selective Agar Media

Selective Medium	Characteristic Colonial Morphology	Gram Stain
Vogel-Johnson Agar Medium	Black surrounded by yellow zones	Positive cocci (in clusters)
Mannitol-Salt Agar Medium	Yellow colonies surrounded by yellow zone	Positive cocci (in clusters)
Baird-Parker Agar Medium	Black, shiny colonies surrounded by clear zones 2 to 5 mm	Positive cocci (in clusters)

Table 7 Morphology and Diagnostic Characteristics of *Pseudomonas aeruginosa* on Selective Agar Media

Selective Medium	Characteristic Colonial Morphology	Fluorescence in UV Light	Oxidase Test	Gram Stain
Cetrimide Agar Medium	Generally greenish	Greenish	Positive	Negative rods
Pseudomonas Agar Medium for Detection of Fluorescin	Generally colourless to yellowish	Yellowish	Positive	Negative rods
Pseudomonas Agar Medium for Detection of Pyocyanin	Generally greenish	Blue	Positive	Negative rods

contained in Petri dishes. Cover and invert the inoculated media, and incubate at 35° ± 2° for not less than 3 days. Examine the streaked surfaces under UV light. Examine the plates to determine whether colonies having the characteristics listed in Table 7 are present.

Confirm any suspect colonial growth on one or more of the media as *Pseudomonas aeruginosa* by means of the oxidase test. Upon the colonial growth, place or transfer colonies to strips or disks of filter paper that previously has been impregnated with *N,N*-dimethyl-*p*-phenylenediamine dihydrochloride. If there is no development of a pink colour, changing to purple, the sample meets the requirements of the test for the absence of *Pseudomonas aeruginosa*. The presence of *Pseudomonas aeruginosa* may be confirmed by other suitable cultural and biochemical tests, if necessary.

BACILLUS ANTHRACIS

Add 10 g (or 10 ml) of the sample to 100 ml of Fluid Soybean-Casein Digest , mix, and incubate at 30° to 35° for 24 to 48 hours. Examine the medium for growth of sporeforming Gram-positive bacilli, with square or concave ends, arranged in long chain, which is typical microscopic morphology of *Bacillus anthracis*. If such growth is present, with the aid of an inoculating loop streak a portion of the medium on the surface of 5 Per Cent Defibrinated Sheep Blood Agar Medium (Medium XXIV) plate and of Egg-Yolk Agar Medium (Medium XXVI) plate, and incubate at 30° to 35° for 18 to 48 hours. If, upon examination, none of the plate contains colonies having the characteristics listed in Table 8 for the media used, the test sample meets the requirements for the absence of *Bacillus anthracis*.

If there are colonies having the characteristics listed in Table 8, further tests should be performed to confirm the identity of *Bacillus anthracis*, including virulent test of anthrax culture either *in vivo* or *in vitro*.

Test for Virulence of *Bacillus anthracis*

In vivo Inject 0.5 ml of supernatant fluid from a 24-hour culture subcutaneously into the thigh of two mice. After 24 hours dissect the infected mice and look for bacteria from organs (spleen, liver) using Gram stain and capsule stain. Fix the preparation with a 0.1 per cent w/v solution of *mercury(II) chloride* instead of heat.

Virulent strains cause a specific generalized infection (anthrax) with encapsulated bacilli in blood and tissues.

In vitro By means of an inoculating loop, transfer representative suspect colonies from the Blood Agar or Egg-Yolk Agar plate onto Soybean-Casein Digest Agar Medium with added 0.5 per cent *sodium hydrogencarbonate* and 0.7 per cent *bovine serum albumin*. Incubate in 5 per cent carbon dioxide or a candle jar at 35° to 37°. Virulent strains become encapsulated, resulting in mucoid colonies.

PATHOGENIC CLOSTRIDIA

Add 10 g (or 10 ml) of the sample being examined into two suitable containers, each containing 100 ml of Cooked-Meat Medium (Medium XXIII), previously heated just prior to use, to 100° for a few minutes and cooled to 37°, or Reinforced Medium for Clostridia (Medium XXIX). To distinguish between sporing and non-sporing organisms, immediately seal one container with a layer of sterile liquid paraffin or agar. Heat the other container at 80° for 10 minutes, cool rapidly, and then similarly seal. Incubate both containers at 35° to 37° and examine every 24-hour period up to 4 days.

After incubation, make subcultures from each container on plates of Columbia Agar (Medium XXX) to which gentamicin has been added and incubate under anaerobic conditions at 35° to 37° for 48 hours. If no growth occurs, the sample passes the test for absence of pathogenic Clostridia.

Table 8 Characteristics of *Bacillus anthracis*

Medium	Characteristic Colonial Morphology	Gram Stain
5 Per Cent Defibrinated Blood Agar Medium	No hemolysis after 24 hours	Gram-positive bacilli with square or concave ends
Egg-Yolk Agar Medium	No ring of white precipitate around colonies	Gram-positive bacilli with square or concave ends

Where growth occurs, subculture each distinct colony form on plates of Columbia Agar Medium, without gentamicin, and incubate at 35° to 37° for 48 hours, one plate anaerobically and the other aerobically, to check that the organism will not grow under aerobic condition.

Examine the appearance of only anaerobic growth of Gram-positive bacilli giving a negative catalase reaction together with the extent of hemolysis, by making subculture on a plate of 5 Per Cent Defibrinated Sheep Blood Agar Medium, and also examine microscopically for spore formation, using Gram stain or spore stain technique and confirmed by further suitable biochemical and biological tests. The description in Table 9 gives the characteristics of some pathogenic Clostridia species on 5 Per Cent Defibrinated Blood Agar Medium.

Clostridium botulinum By means of an inoculating loop, streak representative suspect colonies from the Blood Agar Plate on the surface of Egg-Yolk Agar Medium and incubate at 35° to 37° in an anaerobic jar for 24 hours. If, upon examination, no colonies with opalescence and pearly layer surrounding the colony are observed, the test sample meets the requirements of the test for the absence of *Clostridium botulinum* (Table 10). The presence of *Clostridium botulinum* may be confirmed by further suitable biochemical and biological tests.

Animal inoculation and protection test Botulism may be demonstrated in the guinea-pig following the intraperitoneal inoculation of a fluid culture or culture-filtrate.

Procedure The filtrate is prepared from a 10-day old Cooked-Meat Medium culture which has been incubated at 35° to 37°. A pair of guinea-pigs are infected, one of them having been protected with 0.5 ml of a *polyvalent botulinum antitoxin* at least 1 hour earlier. After an incubation period of a few hours, the earliest symptom of dyspnea is usually developed, the respiration being mainly costal. Thereafter various flaccid paralyses develop, or a

Table 9 Characteristics of *Clostridium* Species on 5 Per Cent Defibrinated Blood Agar Medium

Selective Species	Colonies	Hemolysis	Spores
Clostridium botulinum	Irregular, translucent with a granular surface and in-definited fimbriated spreading edge.	+	Oval, central, subterminal distend bacilli
Clostridium perfringens	Large, circular, convex, semitranslucent, smooth with an entire edge.	Double zone	Oval and subterminal (very rare)
Clostridium tetani	Transparent with long feathery spreading projections	+	Spherical and terminal (drumstick)

Table 10 Characteristics of *Clostridium* Species on Some Media

Clostridium spp. / Media	*Clostridium botulinum*	*Clostridium perfringens*	*Clostridium tetani*
Cooked-Meat Medium	No digestion of meat; much gas, white sediment	No digestion of meat; meat turns pink colour	No digestion of meat; burnt organic smell
Iron-Milk Medium, Modified	Clot formation	Stormy* fermentation	No change
Egg-Yolk Agar Medium	Opalescent zone (Wide zone)*	Opalescent zone (Wide zone)*	No opalescent zone
	Pearly layer (Narrow zone)*	No pearly layer	No pearly layer

* Indicates characteristic of species.

generalized paralysis may result so that the animal lies motionless with limbs outstretched. Death ensues in 18 to 24 hours.

Clostridium perfringens With the aid of an inoculating loop, transfer representative suspect colonies from the Blood Agar plate to a tube containing Fluid Thioglycolate Medium (Medium XXV). Incubate at 35° to 37° until vigorous growth is observed. Then inoculate Iron-Milk Medium (Medium XXVI), using a long tube, and Egg-Yolk Agar Medium, with the culture. For Iron-Milk Medium, incubate at 46° in a water-bath and check hourly after 2 hours for up to 5 hours for stormy fermentation.

(Note This reaction is characterized by rapid coagulation of the milk followed by fracturing of the curd into a spongy mass which usually rises above the medium surface. For this reason, do not use short tubes for the test.) For the Egg-Yolk Agar Medium plate, incubate in an upright position in an anaerobic jar at 35° to 37° for 24 hours. If upon examination, neither the tube nor the plate contains growth having the characteristics listed in Table 10, for the media used, the test sample meets the requirements of the test for the absence of *Clostridium perfringens*. The presence of *Clostridium perfringens* may be confirmed by further suitable biochemical tests.

Clostridium tetani With the aid of an inoculating loop, transfer representative suspect colonies from the Blood Agar plate to a tube containing Cooked-Meat Medium. Immediately seal the tube with a layer of sterile paraffin or agar and incubate at 35° to 37° for 48 hours. If no burnt-organic smell and drumstick appearance are observed, the sample meets the requirements of the test for the absence of *Clostridium tetani*. The presence of *Clostridium tetani* may be confirmed by biochemical or biological tests.

Animal inoculation and protection test The mouse is a suitable laboratory animal for the demonstration of the toxigenicity of *Clostridium tetani*. Two animals are used for each test.

Procedure A protected animal is prepared by subcutaneous injection with 0.5 ml of *tetanus antitoxin* containing 550 units per ml, at least 1 hour before it is inoculated with the virulent organism. Both the protected and the unprotected animals are then inoculated intramuscularly in the right hind limb with 0.25 ml of the supernatant from a 48-hour Cooked-Meat Medium culture of the organism. It may be necessary to include 2.5 per cent calcium chloride in the inoculum to initiate necrosis. After an incubation period of a few hours, signs of tetanus develop in the unprotected mouse.

In ascending tetanus the first evidence of disability is usually that the inoculated leg tends to slip backwards as the animal moves forward. Later the limb becomes slightly abducted and the ankle is extended. The leg gradually becomes more extended until it is quite stiff from the spasm of opposing muscles. The tail of the mouse becomes stiff, and gradually the leg on the opposite side is affected. Involvement of the trunk muscles leads to hyperextension of lateral flexion of the spine and finally the fore limbs become spastic. During this period the slightest stimulus induces a generalized spasm.

In mixed ascending and descending tetanus, which is produced by a large inoculum, local tetanus of the injected limb is followed rapidly by generalized spasms, and death occurs in 18 to 24 hours. Descending tetanus in small laboratory animals is very rare unless special routes of inoculation are used.

Post mortem There is a slight hyperemia at the site of inoculation. The internal organs show little change. The animal protected with tetanus antitoxin shows no evidence of infection.

Retest

For the purpose of confirming a doubtful result by and of the procedures outlined in the foregoing tests following their application to a 10.0-g sample, a retest or a 25-g sample may be conducted. Proceed as directed under *Preparation of Sample*, but make allowance for the larger sample size.

10.5 LIMITS FOR MICROBIAL CONTAMINATION pp. 1573-1574

Change to read:

10.5 LIMITS FOR MICROBIAL CONTAMINATION

In the manufacture, packaging, storage and distribution of pharmaceutical preparations, suitable means must be taken to ensure their microbiological quality. Unless otherwise specified in the individual monograph, the non-sterile pharmaceutical preparations should comply with the criteria given below.

Category	Types	Requirements*
1	Preparations for burns and severe ulcerations.	- Absence of viable micro-organisms per g or per ml.
2	Topical preparations for broken skins, abscess, lesions, and mucous membranes (nose, throat, ear, vagina, etc.).	- Total viable aerobic count. Not more than a total of 5×10 micro-organisms (aerobic bacteria plus fungi) per g or per ml. - Absence of enterobacteria and certain other Gram-negative bacteria, *Pseudomonas aeruginosa* and *Staphylococcus aureus* per g or per ml.
3	Transdermal patches and topical preparations for intact skin, e.g., creams, lotions, ointments, solutions, powders, etc.	- Total viable aerobic count. Not more than a total of 5×10^2 micro-organisms (aerobic bacteria plus fungi) per g or per ml or per patch. - Absence of enterobacteria and certain other Gram-negative bacteria, *Pseudomonas aeruginosa* and *Staphylococcus aureus* per g or per ml or per patch.
4	A. Preparations for oral and rectal administration.	- Total viable aerobic count. Not more than 5×10^3 bacteria and not more than 5×10^2 fungi per g or per ml. - Not more than 10^2 enterobacteria and certain other Gram-negative bacteria per g or per ml. - Absence of *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Escherichia coli* per g or per ml. - Absence of *Salmonella* spp. per 10 g or per 10 ml.
	B. Preparations for oral administration containing raw materials of natural origin (animal, vegetable or mineral) which cannot be treated with a process for reduction of microbial count. Herbal remedies described in categories 5 and 6 are excluded.	- Total viable aerobic count. Not more than 5×10^4 bacteria and not more than 5×10^2 fungi per g or per ml. - Not more than 10^2 enterobacteria and certain other Gram-negative bacteria per g or per ml. - Absence of *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Escherichia coli* per g or per ml. - Absence of *Salmonella* spp. per 10 g or per 10 ml.
5	Preparations of crude drugs and mixtures of crude drugs for internal use which will undergo a process for reduction of microbial count before use (e.g., by pouring boiling water over them); and preparations for external use containing whole or ground crude drugs.	- Total viable aerobic count. Not more than 5×10^7 bacteria and not more than 5×10^4 fungi per g or per ml. - Not more than 10^4 enterobacteria and certain other Gram-negative bacteria per g or per ml. - Absence of *Escherichia coli* per g or per ml. - Absence of *Salmonella* spp, *Bacillus anthracis***, and pathogenic *Clostridium* spp. per 10 g or per 10 ml.

Category	Types	Requirements*
6	Other preparations for internal use containing whole or ground crude drugs.	- Total viable aerobic count. Not more than 5×10^5 bacteria and not more than 5×10^3 fungi per g or per ml. - Not more than 10^3 enterobacteria and certain other Gram-negative bacteria per g or per ml. - Absence of *Escherichia coli* and *Staphylococcus aureus* per g or per ml. - Absence of *Salmonella* spp. *Bacillus anthracis***, and pathogenic *Clostridium* spp., per 10 g or per 10 ml.

* Carry out the tests as described in the "Microbial Limit Tests" (Appendix 10.2).

** Applied only when anthrax outbreaks are reported.

10.6 EFFICACY OF ANTIMICROBIAL PRESERVATION pp. 2065-2066

Change to read:

10.6 EFFICACY OF ANTIMICROBIAL PRESERVATION

Antimicrobial preservatives are substances added to pharmaceutical preparations to prevent microbial proliferation or to limit microbial contamination that may occur during normal conditions of storage and use. They are used primarily in multiple-dose parenteral, ear, oral, nasal topical and eye preparations made with aqueous bases or vehicles. Antimicrobial preservatives should not be used as a substitute for good manufacturing practices or solely to reduce the viable microbial population of a nonsterile product or control the presterilization bioburden of multidose formulations during manufacturing.

The efficacy of an antimicrobial preservative may be enhanced or diminished by many factors. Those include the active ingredient, the formulation, and the container or closure used for that product. The test for efficacy of antimicrobial preservation should therefore be carried out on the product as presented, wherever possible in its original, unopened container in which it was distributed by the manufacturer.

During development of a pharmaceutical preparation, it should be demonstrated that the antimicrobial activity of the preparation provides adequate protection from microbial contamination during storage and use. The test described below is therefore designed to determine the efficacy of antimicrobial activity of the product. The test is not intended to be performed on a routine control basis.

The test for efficacy of antimicrobial preservation in the preparation consists of challenging the preparation with a prescribed inoculum of suitable micro-organisms, storing the inoculated preparation at a prescribed condition, and withdrawing samples from the container at prescribed time intervals to count the remaining viable organisms.

The preservative properties of the preparation are adequate if, in the conditions of the test, there is a significant decrease or no increase in the number of micro-organisms in the inoculated preparation after the times and at the temperatures prescribed. The criteria of acceptance vary for different types of preparation according to the degree of protection intended.

Product Categories

For the purpose of testing, products have been divided into four categories (see Table 1). The criteria of antimicrobial effectiveness for these products are a function of the route of administration.

Test Organisms

Aspergillus niger	ATCC[1] 16404, DMST[2] 15538
Candida albicans	ATCC 10231, DMST 5812
Escherichia coli	ATCC 8739, DMST 15537
Pseudomonas aeruginosa	ATCC 9027, DMST 15501
Staphylococcus aureus	ATCC 6538, DMST 8013

Single-strain challenges, either ATCC or DMST, should be used throughout the test.

[1] ATCC = American Type Culture Collection
[2] DMST = Department of Medical Sciences, Thailand

Table 1 Product Categories

Category	Product Description
1	Injections, other parenterals including emulsions, ear preparations, sterile nasal preparations, and eye preparations made with aqueous bases or vehicles.
2	Topically used preparations made with aqueous bases or vehicles, nonsterile nasal preparations, and emulsions, including those applied to mucous membranes.
3	Oral preparations other than antacids, made with aqueous bases or vehicles.
4	Antacids made with an aqueous base.

Media

For the initial cultivation of the test organisms, select an agar medium that promotes vigorous growth of the respective stock culture, such as Soybean-Casein Digest Agar Medium for bacteria and Sabouraud Dextrose Agar Medium for fungi (Appendix 10.2).

Preparation of Inoculum

Begin the test by inoculating the surface of a suitable solid agar medium from the recently grown stock culture of each of the specified micro-organisms. Incubate the bacterial cultures at 30° to 35° for 18 to 24 hours, the culture of *Candida albicans* at 20° to 25° for 48 hours, and the culture of *Aspergillus niger* at 20° to 25° for 1 week, or until good sporulation is obtained.

To harvest the bacterial and *Candida albicans* cultures, use a sterile 0.9 per cent w/v solution of *sodium chloride* for dispersal and transfer of the surface growth into a suitable vessel. Add sufficient suspending fluid to reduce the microbial count to about 1×10^8 colony-forming units (cfu) per ml. To harvest the *Aspergillus niger* culture, use a sterile 0.9 per cent w/v solution of *sodium chloride* containing 0.05 per cent w/v of *polysorbate 80* and adjust the spore count to about 1×10^8 cfu per ml by adding the same solution.

Alternatively, the stock culture organisms may be grown in a suitable liquid medium, and the micro-organisms may be harvested by centrifugation, washed, and dispersed in a sterile 0.9 per cent w/v solution of *sodium chloride* to give the required microbial or spore count.

Determine immediately the number of colony-forming units per ml in each suspension by means of Plate Method (Appendix 10.2). This value serves to calibrate the size of inoculum used in the test. The bacterial and yeast suspensions are to be used within 24 hours of harvest, but the fungal preparation may be stored under refrigeration for up to seven days.

Procedure

To count the viable micro-organisms in the inoculated preparations, use the agar medium corresponding to that used for the initial cultivation of the respective micro-organisms. Ensure that any residual antimicrobial activity of the products is eliminated either by dilution, by filtration or by the use of a specific inactivator.

If the product container can be entered aseptically, with needle and syringe through a stopper, conduct the test in five original containers. If not, transfer a 20-ml portion of preparation from five containers to each of five sterile capped tubes. Inoculate each container with one of the prepared and standardized inoculum, and mix. The volume of the suspension inoculum used is between 0.5 per cent and 1.0 per cent of the volume of the product. The concentration of test micro-organisms that is added to the product (Categories 1, 2, and 3) are such that the final concentration of the test preparation after inoculation is between 1×10^5 and 1×10^6 cfu per ml of the product. For Category 4 products (antacids) the final concentration of the test preparation after inoculation is between 1×10^3 and 1×10^4 cfu per ml of the product. Determine the number of viable micro-organisms in each inoculated suspension and calculate the initial concentration of micro-organisms per ml of preparation.

Incubate the inoculated containers or tubes at 20° to 25°. Sample each container at the

appropriate intervals specified in Table 2. Record any changes observed in appearance at these intervals. Determine by the Plate Method the number of cfu present in each test preparation for the applicable intervals. Using the calculated concentrations of cfu per ml present at the start of the test, calculate the change in $\log_{10}$ values of the concentration of cfu per ml for each micro-organism at the applicable test intervals, and express the changes in terms of log reductions.

Evaluation

The criteria for evaluation of antimicrobial activity are given in Table 2 in terms of the log reduction in the number of viable micro-organisms. No increase (NI) is defined as not more than 0.5 $\log_{10}$ unit higher than the previous value measured.

Table 2 Criteria for the Evaluation of Preservative Efficacy

For Category 1 Products			
Test Organisms	Log Reduction		
	7 days	14 days	28 days
Bacteria	≥ 1.0	≥ 3.0	NI
Yeast and Molds	NI	NI	NI

For Category 2 Products		
Test Organisms	Log Reduction	
	14 days	28 days
Bacteria	≥ 2.0	NI
Yeast and Molds	NI	NI

For Category 3 Products		
Test Organisms	Log Reduction	
	14 days	28 days
Bacteria	≥ 1.0	NI
Yeast and Molds	NI	NI

For Category 4 Products		
Test Organisms	Log Reduction	
	14 days	28 days
Bacteria	NI	NI
Yeast and Molds	NI	NI

APPENDIX 12 STERILIZATION AND STERILITY ASSURANCE

Sterilization is the process of rendering an article, or product, free from viable micro-organisms. It may be effected by killing the micro-organisms by physical or chemical methods or by removing them by filtration. Wherever possible, a process in which the product is sterilized in its final container (terminal sterilization) is chosen. If terminal sterilization is not possible, filtration through a bacteria-retentative filter or aseptic processing is used.

The method of attaining sterility in an article is determined by the nature of the product, the extent and type of any contamination present and the conditions under which the product has been prepared; it is assumed that the principles of good manufacturing practice will have been observed. Materials to be sterilized should be as free as possible from microbial contamination. The effect of the chosen sterilization process on the product (including its final container or package) should be validated before that procedure is applied in practice. Failure to follow a process meticulously involves the risk of a non-sterile or deteriorated product. Proper validation of the sterilization process or the aseptic process requires, however, a high level of knowledge of the field of sterilization and clean room technology. In order to comply with currently acceptable and achievable limits in sterilization parameters, it is necessary to employ appropriate instrumentation and equipment to control the critical parameters such as temperature and time, humidity, and sterilizing gas concentration, or absorbed radiation. An important aspect of the validation programme in many sterilization procedures involves the employment of biological indicators. The validated and certified process should be revalidated periodically; however, the revalidation programme need not necessarily be as extensive as the original programme.

Within the strictest definition of sterility, an article would be deemed sterile only when there is complete absence of viable micro-organisms from it. However, this absolute definition cannot currently be applied to an entire lot of finished compendial articles because of limitations in testing. Absolute sterility cannot be practically demonstrated without complete destruction of every finished article. The sterility of a lot purported to be sterile is therefore defined in probabilistic terms, where the likelihood of a contaminated unit or article is acceptably remote. Such a state of sterility assurance can be established only through the use of adequate sterilization cycles and subsequent aseptic processing, if any, under appropriate current good manufacturing practice, and not by relying solely on sterility testing.

Sterility assurance level (SAL) The SAL of a sterilizing process is the degree of assurance with which the process in question renders a population of items sterile. The SAL for a given process is expressed as the probability of a non-sterile item in that population. An SAL of 10^{-6}, for example, denotes a probability of not more than one viable micro-organism in 1×10^{6} sterilized items of the final product. The SAL of a process for a given product is established by appropriate validation studies.

Methods of Sterilization

Methods of terminal sterilization, including removal of micro-organism by filtration, and aseptic processing are described. Modern technological developments, however, have led to the use of additional procedures. The choice of the appropriate process for a given dosage form or component requires a high level of knowledge of sterilization techniques and information concerning any effects of the process on the material being sterilized.

Steam sterilization The process of thermal sterilization employing saturated steam under pressure is carried out in a chamber called an autoclave. It is probably the most widely employed sterilization process, especially for aqueous preparation. The basic principle of operation is that the air in the sterilizing chamber is displaced by the saturated steam, achieved by employing vents or traps. In order to displace air more effectively from the chamber and from within articles, the sterilization cycle may include air and steam evacuation stages.

For this method of terminal sterilization, the reference conditions for aqueous preparations are heating at a minimum of 121° for 15 minutes. Other combinations of time and temperature may be used provided that it has been satisfactorily demonstrated that the process chosen delivers an adequate and reproducible level of lethality when operating routinely within the established tolerances. The procedures and precautions employed are such as to give an SAL of 10^{-6} or better. A biological assessment of the process may be obtained by including a suitable biological indicator.

With large batch sizes of aqueous preparations, it is essential to have knowledge of the physical conditions within the autoclave chamber during the sterilization procedure. To obtain this information, recording temperature-sensitive elements inserted into representative containers may be used together with additional elements at the previously-established coolest part of the loaded chamber. It is desirable that each sterilization cycle be recorded on a temperature-time chart. Other types of temperature indicator may be inserted at appropriate positions in the load but total reliance should not be placed on chemical indicators except when they suggest failure to attain sterilizing conditions.

When surgical dressings are sterilized by Steam sterilization, the steam used should not contain more than 5 per cent of entrained moisture. Most dressings are sterilized by maintaining at a temperature of 134° to 138° for 3 minutes, but other suitable combinations of temperature and time may be used, the conditions being chosen with regard to the stability of the dressings.

Apart from that description of sterilization cycle parameters, using a temperature of 121°, the F_0 concept may be appropriate. The F_0, at a particular temperature other than 121°, is the time (in minutes) required to provide the lethality equivalent to that provided at 121° for a stated time.

The total F_0 of a process takes account of the heating up and cooling down phases of the cycle and can be calculated by integration of lethal rates with respect to time at discrete temperature intervals.

When a steam sterilization cycle is chosen on the basis of the F_0 concept, great care must be taken to ensure that an adequate assurance of sterility is consistently achieved. In addition to validating the process, it may also be necessary to perform continuous, rigorous microbiological monitoring during routine production to demonstrate that the microbiological parameters are within the established tolerances so as to given as SAL of 10^{-6} or better.

In connection with sterilization by steam, the Z-value relates the heat resistance of a micro-organism to changes in temperature. The Z-value is the change in temperature required to alter the D-value by a factor of 10.

The D-value (or decimal reduction value) is the value of a parameter of sterilization (duration or absorbed dose) required to reduce the number of viable organisms to 10 per cent of the original number. It is only of significance under precisely defined experimental conditions.

The following mathematical relationships apply:

$$F_0 = D_{121}(\log N_0 - \log N) = D_{121} \log IF$$

where D_{121} = D-value of the reference spores at 121°,

N_0 = initial number of viable micro-organisms,

N = final number of viable micro-organisms,

IF = inactivation factor.

$$Z = (T_2 - T_1)/(\log D_1 - \log D_2)$$

where D_1 = D-value of the micro-organism at temperature T_1,

D_2 = D-value of the micro-organism at temperature T_2,

$$IF = N_0/N = 10^{t/D}$$

where t = exposure time,

D = D-value of micro-organism in the exposure conditions.

Dry-heat sterilization Dry-heat sterilization may be used for heat stable non-aqueous preparations, powders and certain impregnated dressings. For this method of terminal sterilization the reference conditions are a minimum of 160° for at least 2 hours. Other combinations of time and temperature may be used provided that it has been satisfactorily demonstrated that the process chosen delivers an adequate and reproducible level of lethality when operated routinely within the established tolerances. The procedures and precautions employed are such as to give an SAL of 10^{-6} or better. A modern oven is supplied with heated, filtered air, distributed uniformly throughout the chamber by convection or radiation and employing a blower system with devices for sensing, monitoring, and controlling the critical parameters.

Appropriate biological indicators may be employed to demonstrate the effectiveness of the sterilization process. An example of a biological indicator for validating and monitoring dry-heat sterilization is a preparation of *Bacillus subtilis*

spores. For heat-stable articles or components, the conditions of sterilization are not less than 250°. A microbial survival probability of 10^{-12} is considered achievable for heat-stable articles or components. Since dryheat at 250° is frequently employed to render glassware or containers free from pyrogens as well as viable microbes, a pyrogen challenge, where necessary, should be an integral part of the validation program, e.g., by inoculating one or more of the articles to be treated with 1000 or more EU of bacterial endotoxin. The test with *Limulus* lysate could be used to demonstrate that the endotoxic substance has been inactivated to not more than 1/1000 of the original amount (3 log cycle reduction). For the test to be valid, both the original amount and, after acceptable inactivation, the remaining amount of endotoxin should be measured. For additional information on the endotoxin assay, see under the "Test for Bacterial Endotoxins" (Appendix 8.5) .

Gas sterilization This method of sterilization is only to be used where there is no suitable alternative. It is essential that penetration by gas and moisture into the material to be sterilized is ensured and that it is followed by a process of elimination of the gas under conditions that have been previously established to ensure that any residue of gas or its transformation products in the sterilized product is below the concentration that could give rise to toxic effects during use of the product. The active agent generally employed in gaseous sterilization is ethylene oxide of acceptable sterilizing quality or a mixture of ethylene oxide with a suitable inert gas.

Wherever possible, the gas concentration, relative humidity, temperature and duration of the process are measured and recorded. Measurements are made where sterilization conditions are least likely to be achieved, as determined at validation.

The effectiveness of the process applied to each sterilization load is checked using a suitable biological indicator.

Ionizing radiation sterilization Sterilization by this method is achieved by exposure of the product to ionizing radiation in the form of gamma radiation from a suitable radioisotopic source, such as cobalt-60 (^{60}Co) or of a beam of electrons energized by a suitable electron accelerator.

For this method of terminal sterilization the reference absorbed dose is 25 kGy. Other doses may be used provided that it has satisfactorily been demonstrated that the dose chosen delivers an adequate and reproducible level of lethality when the process is operated routinely within the established tolerances. The procedures and precautions employed are such as to give an SAL of 10^{-6} or better.

During the sterilization procedure the radiation absorbed by the product is monitored regularly by means of established dosimetry procedures that are independent of dose rate. Dosimeters are calibrated against a standard source at a reference radiation plant on receipt from the supplier and at suitable intervals of not longer than one year thereafter.

Where a biological assessment is carried out, this is obtained using a suitable biological indicator.

Filtration Sterilization by Filtration may be used for certain medicaments and preparations which are not sufficiently stable to heat to allow sterilization by steam sterilization. Solutions or liquids may be sterilized by passage through a sterile bacteria-retaining filter of a type that has been demonstrated to be satisfactory by means of a microbial challenge test using a suitable test micro-organism. A suspension of *Pseudomonas diminuta* (ATCC 19146, NCIMB 11091 or CIP 103020) may be suitable. It is recommended that a challenge of at least 10^7 cfu per cm^2 of active filter surface is used and that the suspension is prepared in Soybean-Casein Digest Medium which, after passage through the filter, is collected aseptically and incubated aerobically at 30° to 35°. Such products need special precautions. The production process and environment are regularly subjected to appropriate monitoring procedures. The equipment, containers and closures and, wherever possible, the ingredients are subjected to an appropriate sterilization process. It is recommended that the filtration process be carried out as close as possible to the filling point. The operations following filtration are carried out under aseptic conditions.

Filtration for sterilization is usually carried out with assemblies having membranes of porosity not greater than 0.22 µm; however, membranes of smaller porosities are also used and may be needed for some products. The types of membrane filter which are now available include cellulose acetate, cellulose nitrate, fluorocarbonate, acrylic polymers, polycarbonate, polyester, polyvinyl chloride, and even metal membranes, and they may be reinforced or supported by an internal fabric. A membrane

filter assembly should be tested for integrity of the membrane and its effectiveness confirmed before and after use. A typical test is the bubble-point test, whereby it is determined that a prescribed pressure is necessary to force air bubbles through the intact membrane wetted with either product, water or hydrocarbon liquid.

Biological Indicators

Biological indicators are standardized preparations of selected micro-organisms used to assess the effectiveness of a sterilization procedure. They usually consist of a population of bacterial spores placed on an inert carrier, for example a strip of filter paper, a glass slide or a plastic tube. The inoculated carrier is covered in such a way that it is protected from any deterioration or contamination, while allowing the sterilizing agent to enter into contact with the micro-organisms. Spore suspensions may be presented in sealed ampoules. Biological indicators are prepared in such a way that they can be stored under defined conditions; an expiry date is set. Micro-organisms of the same bacterial species as the bacteria used to manufacture the biological indicators may be inoculated directly into a liquid product to be sterilized or into a liquid product similar to that to be sterilized. In this case, it must be demonstrated that the liquid product has no inhibiting effect on the spores used, especially as regards their germination.

A biological indicator is characterized by the name of the species of bacterium used as the reference micro-organism, the number of the strain in the original collection, the number of viable spores per carrier and the D-value. The D-value is the value of a parameter of sterilization (duration or absorbed dose) required to reduce the number of viable organisms to 10 per cent of the original number. It is of significance only under precisely defined experimental conditions. Only the stated micro-organisms are present. Biological indicators consisting of more than one species of bacteria on the same carrier may be used. Information on the culture medium and the incubation conditions is supplied. It is recommended that the indicator organisms be placed at the locations presumed, or wherever possible, found by previous physical measurement to be least accessible to the sterilizing agent. After exposure to the sterilizing agent, aseptic technique is used to transfer carriers of spores to the culture media, so that no contamination is present at the time of examination. Biological indicators that include an ampoule of culture medium placed directly in the packaging protecting the inoculated carrier may be used.

A choice of indicator organisms is made such that:

(a) the resistance of the test strain to the particular sterilization method is great compared to the resistance of all pathogenic micro-organisms and to that of micro-organisms potentially contaminating the product;

(b) the test strain is non-pathogenic;

(c) the test strain is easy to culture.

After incubation, growth of the reference micro-organisms subjected to the sterilization procedure demonstrates that this procedure is unsatisfactory.

Steam sterilization The use of biological indicators intended for steam sterilization is recommended for the validation of sterilization cycles. Spores of *Bacillus stearothermophilus* (for example, ATCC 7953, NCTC 10007, NCIMB 8157 or CIP 52.81) are recommended. The number of viable spores exceeds 5×10^5 per carrier. The D-value at 121° exceeds 1.5 minutes. It is verified that exposing the biological indicators to steam at 121° ± 1° for 6 minutes leaves revivable spores, and that there is no growth of the reference micro-organisms after the biological indicators have been exposed to steam at 121° ± 1° for 15 minutes.

Dry-heat sterilization Spores of *Bacillus subtilis* (for example, var. niger ATCC 9372, NCIMB 8058 or CIP 77.18) are recommended for the preparation of biological indicators. The number of viable spores exceeds 1×10^5 per carrier and the D-value at 160° is approximately 5 to 10 minutes. Dry heat at 250° is frequently used for sterilization and depyrogenation of glassware. In this case, demonstration of a 3 log reduction in heat resistant bacterial endotoxin can be used as a replacement for biological indicators.

Gas sterilization The use of biological indicators is necessary for all gas sterilization procedures, both for the validation of the cycles and for routine operations. The number of viable spores exceeds 5×10^5 per carrier. For hydrogen peroxide and peracetic acid spores of *Bacillus stearothermophilus* (for example ATCC 7953, NCTC 10007, NCIMB 8157 or CIP 52.81), for ethylene oxide and formaldehyde spores of *Bacillus subtilis* (for example, var. niger ATCC 9372, NCIMB 8058 or CIP

77.18) are recommended. The parameters of resistance are known for the procedure used: for example, for ethylene oxide, the D-value exceeds 2.5 minutes for a test cycle involving 600 mg per litre of ethylene oxide, at 54° and at 60 per cent relative humidity. It is verified that there is no growth of the reference micro-organisms after the biological indicators have been exposed to the test cycle described above for 60 minutes and that exposing the indicators to a reduced temperature cycle (600 mg per litre at 30° and 60 per cent relative humidity) for 15 minutes leaves revivable spores. It is essential that the biological indicator be able to reveal insufficient humidification in the sterilizer and the product to ensure dehydrated micro-organisms are inactivated. Exposing the indicators to 600 mg per litre of ethylene oxide at 54° for 60 minutes without humidification must leave revivable spores.

Ionizing radiation sterilization Biological indicators may be used to monitor routine operations, as an additional possibility to assess the effectiveness of the set dose of radiation energy, especially in the case of accelerated electron sterilization. The spores of *Bacillus pumilus* (for example, ATCC 27.142, NCTC 10327, NCIMB 10692 or CIP 77.25) are recommended. The number of viable spores exceeds 1×10^7 per carrier. The D-value exceeds 1.9 kGy. It is verified that there is no growth of the reference micro-organisms after the biological indicators have been exposed to 25 kGy (minimum absorbed dose).

Aseptic Processing

While there is general agreement that sterilization of the final filled container as a dosage form or final packaged device is the preferred process for assuring the minimal risk of microbial contamination in a lot, there is a substantial class of products that are not terminally sterilized but are prepared by a series of aseptic steps. These are designed to prevent the introduction of viable micro-organisms into components, where sterile, or once an intermediate process has rendered the bulk product or its components free from viable micro-organisms. A review of the principles involved in producing aseptically processed products with a minimal risk of microbial contamination in the finished lot of final dosage forms is hereby provided.

A product defined as aseptically processed is likely to consist of components that have been sterilized by one of the processes described above. For example, the bulk product, if filterable, may have been sterilized by filtration. The final empty container components would probably be sterilized by heat, dry heat being employed for glass vials and an autoclave being employed for rubber closures. The areas of critical concern are the immediate microbial environment where these pre-sterilized components are exposed during assembly to produce the finished dosage form and the aseptic filling operation.

The requirements for a properly designed, validated and maintained filling or other aseptic processing facility are mainly directed to (1) an air environment free from viable micro-organisms, of a proper design to permit effective maintenance of air supply units, and (2) the provision of trained operating personnel who are adequately equipped and gowned. The desired environment may be achieved through the high level of air filtration technology, which contributes to the delivery of air of the requisite microbiological quality. The facilities include both primary (in the vicinity of the exposed article) and secondary (where the aseptic processing is carried out) barrier systems.

For a properly designed aseptic processing facility or aseptic filling area, consideration should be given to such features as nonporous and smooth surfaces, including walls and ceilings that can be sanitized frequently; gowning rooms with adequate space for personnel and storage of sterile garments; adequate separation of preparatory rooms for personnel from final aseptic processing rooms, with the availability where necessary of such devices as airlocks and/or air showers; proper pressure differentials between rooms, the most positive pressure being in the aseptic processing rooms or areas; the employment of laminar (unidirectional) air flow in the immediate vicinity of exposed product or components, and filtered air exposure thereto, with adequate air change frequency; appropriate humidity and temperature environmental controls; and a documented sanitization programme. Proper training of personnel in hygienic and gowning techniques should be undertaken so that, for example, gowns, gloves, and other body coverings substantially cover exposed skin surfaces.

Certification and validation of the aseptic process and facility is achieved by establishing the efficiency of the filtration systems, by employing microbiological environmental monitoring procedures, and by processing of sterile culture

medium as simulated product.

Monitoring of the aseptic facility should include periodic environmental filter examination as well as routine particulate and microbiological environmental monitoring, and may include periodic sterile culture medium processing.

Sterility Testing of Lots

It should be recognized that the referee sterility test might not detect microbial contamination if present in only a small percentage of the finished articles in the lot because the specified number of units to be taken imposes a significant statistical limitation on the utility of the test results. This inherent limitation, however, has to be accepted since current knowledge offers no nondestructive alternatives for ascertaining the microbiological quality of every finished article in the lot, and it is not a feasible option to increase the number of specimens significantly.

The primary means of supporting the claims that a lot of finished articles purporting to be sterile meets the specifications consist of the documentation of the actual production and sterilization record of the lot and of the additional validation records that the sterilization process possesses the capability of totally inactivating the established product microbial burden or a more resistant challenge. Further, it should be demonstrated that any processing steps involving exposed product following the sterilization procedure are performed in an aseptic manner, to prevent contamination. If data derived from the manufacturing process sterility assurance validation studies and from in-process controls are judged to provide greater assurance that the lot meets the required low probability of containing a contaminated unit (compared to sterility testing results from finished units drawn from that lot), any sterility test procedures adopted may be minimal, or dispensed with on a routine basis. However, assuming that all of the above production criteria have been met, it may still be desirable to perform sterility testing on samples of the lot of finished articles. Such sterility testing is usually carried out directly after the lot is manufactured as a final product quality control test. Sterility tests employed in this way in manufacturing control should not be confused with those described under the other section of "Sterility Test" (Appendix 10.1). The procedural details may be the same with regard to media, inocula and handling of specimens, but the number of units and/or incubation time(s) selected for testing may differ. The number should be chosen relative to the purpose to be served, i.e., according to whether greater or lesser reliance is placed on sterility testing in the context of all the measures for sterility assurance in manufacture. Also, longer times of incubation would make the test more sensitive to slow-growing micro-organisms. In the growth promotion tests for media, such slow growers, particularly if isolated from the product microbial burden, should be included with the other test strains.

Negative or satisfactory sterility test results serve only as further support of the existing evidence concerning the quality of the lot if all of the pertinent production records of the lot are in order and the sterilizing or aseptic process is known to be effective. Unsatisfactory test results, however, in manufacturing quality control indicate a need for further action.

Interpretation of quality control tests Quality control sterility tests (either according to the official referee test or modified tests) may be carried out in two separate stages in order to rule out false positive results.

First stage Regardless of the sampling plan used, if no evidence of microbial growth is found, the results of the test may be taken as indicative of absence of intrinsic contamination of the lot.

If microbial growth is found, proceed to the Second stage (unless the First stage test can be invalidated). Evidence for invalidating a First stage test in order to repeat it as a First stage test may be obtained from a review of the testing environment and the relevant records thereto. Finding of microbial growth in negative controls need not be considered the sole grounds for invalidating a First stage test. When proceeding to the Second stage, particularly where depending on the results of the test for lot release, concurrently initiate and document a complete review of all applicable production and control records. In this review consideration should be paid to the following:

a. A check on monitoring records of the validated sterilization cycle applicable to the product.

b. Sterility test history relating to the particular product for both finished and in-process samples, as well as sterilization records of supporting equipment, containers/closures, and sterile components, if any.

c. Environmental control data, including those obtained from media fills, exposure plates, filtering records, any sanitization records and microbial monitoring records of operators, gowns, gloves, and garbing practices.

Failing any lead from the above review, the current microbial profile of the product should be checked against the known historical profile for possible change. Records should be checked concurrently for any changes in source of product components and/or in-processing procedures that might be contributory. Depending on the findings, and in extreme cases, consideration may have to be given to revalidation of the total manufacturing process.

Second stage For the Second stage it is not possible to specify a particular number of specimens to be taken for testing. It is usual to select double the number specified for the First stage under "Sterility Tests" (Appendix 10.1), or other reasonable number. The minimum volumes tested from each specimen, the media, and the incubation periods are the same as those indicated for the First stage.

If no microbial growth is found in the Second stage, and the documented review of appropriate records and the indicated product investigation does not support the possibility of intrinsic contamination, the lot may be considered to meet the requirements of a test for sterility. If growth is found, the lot fails to meet the requirements of the test. As was indicated for the First stage test, the Second stage test may similarly be invalidated with appropriate evidence, and, if so done, repeated as a Second stage test.

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