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Annex 3

General requirements for the sterility of biological substances

(Requirements for Biological Substances No. 6, revised 1973, amendment 1995)

Introduction

Since the General Requirements for the Sterility of Biological Substances were revised in 1973 (*1*), several developments have occurred. It is thus appropriate to modify as follows the existing General Requirements for the Sterility of Biological Substances.

Sterility test for mycoplasmas (section 5.3, page 52)

Replace the text of section 5.3 by the following:

"The test for mycoplasmas is carried out by culture in agar and broth or by means of an indicator cell culture and DNA staining.

Where a test for mycoplasmas is prescribed for a master cell bank, for a working cell bank, for a virus seed lot (or bank) or for cell cultures used as controls, the standard culture or the indicator cell culture method can be used. Where a test for mycoplasmas is prescribed for a virus harvest, bulk vaccine or final lot, the standard culture method is used. The indicator cell-culture method can be used, where necessary, for the screening of media. Both the standard culture and indicator cell-culture methods are given in Appendix 3. Alternative methods may be used provided that they have been validated against the methods described here."

Tests for mycoplasmas (Appendix 3, p. 56)

Replace the text of Appendix 3 by the following:

"Standard culture method

Choice of culture media

The test is carried out with a sufficient number of both solid and liquid media to ensure the growth, under the chosen incubation conditions, of small numbers of organisms that may be present in the product

ability of the media to support the growth of at least the following organisms shall be demonstrated:

Mycoplasma orale (vaccines for human use);

Mycoplasma pneumoniae (vaccines for human use);

Mycoplasma hyorhinis (non-avian veterinary vaccines, e.g. strain DBS 1050);

Mycoplasma hyopneumoniae (non-avian veterinary vaccines);

Mycoplasma gallisepticum and *Mycoplasma synoviae* (where avian material has been used during vaccine production or where the vaccine is intended for use in poultry).

Low-passage test strains are used and are stored frozen or freeze-dried. After cloning, the strains are identified as being of the required species by a suitable method.

In some countries, specific tests for insect mycoplasmas are performed.

Incubation conditions

Divide inoculated media into two equal parts; incubate one in aerobic conditions (an atmosphere of air containing 5–10% carbon dioxide and adequate humidity) and the other in anaerobic conditions (an atmosphere of nitrogen containing 5–10% carbon dioxide and adequate humidity).

Demonstration of nutritive properties of media

Inoculate the media with the appropriate test organisms; use no fewer than 200 and no more than 400 colony-forming units per plate of solid medium and no fewer than 20 and no more than 40 colony-forming

is checked by repeating the test for inhibitory substances after neutralization.

Test for mycoplasmas

For solid media, use plates 60 mm in diameter containing 9 ml of medium. Inoculate each of at least 2 plates of each solid medium with 0.2 ml of the product to be examined and inoculate at least 2 of each

detected by their characteristic particulate or filamentous pattern of fluorescence on cell surfaces and, if contamination is heavy, in surrounding areas.

Verification of the substrate

With a Vero-cell substrate, pretest the procedure with an inoculum of no more than 100 colony-forming units (CFU) of a strain growing readily in liquid or solid media and demonstrate its ability to detect potential mycoplasma contaminants such as *M. hyorhinis* strain DBS 1050 and *M. orale* strain 1596 or other suitable strains. A different cell substrate may be used, e.g. the vaccine-production cell line, if it has been demonstrated that it provides at least equal sensitivity for the detection of mycoplasma contamination.

Test for mycoplasmas

Take no less than 1 ml of the material to be examined and use it to inoculate 2 or more indicator cell cultures grown to 50% confluence and representing no less than 25 cm^2 of cell culture in area; make at least one passage. The product of the last passage is incubated on coverslips placed in suitable containers or on some other suitable surface for the test procedure.

Include in the test a negative (non-inoculated) control and two positive controls inoculated with species such as *M. hyorhinis* and *M. orale*. Use an inoculum of not more than 100 CFU for the positive controls.

If for viral suppressions the interpretation of results is obscured by cytopathic effects, the virus may be neutralized by means of a specific antiserum that has no inhibitory effects on mycoplasmas. A cell culture substrate that does not allow growth of the virus may also be used.

Procedure

(a) Seed the culture at a regular density (2×10^4 to 2×10^5 cells per ml, $4 \times 10^3 - 2.5 \times 10^4$ cells/cm^2) and incubate at 36 ± 1 °C until about 50% confluence is reached. Inoculate the product to be examined and incubate to confluence.

(b) Remove and discard the medium.

(c) Rinse the monolayer with phosphate buffered saline pH 7.4

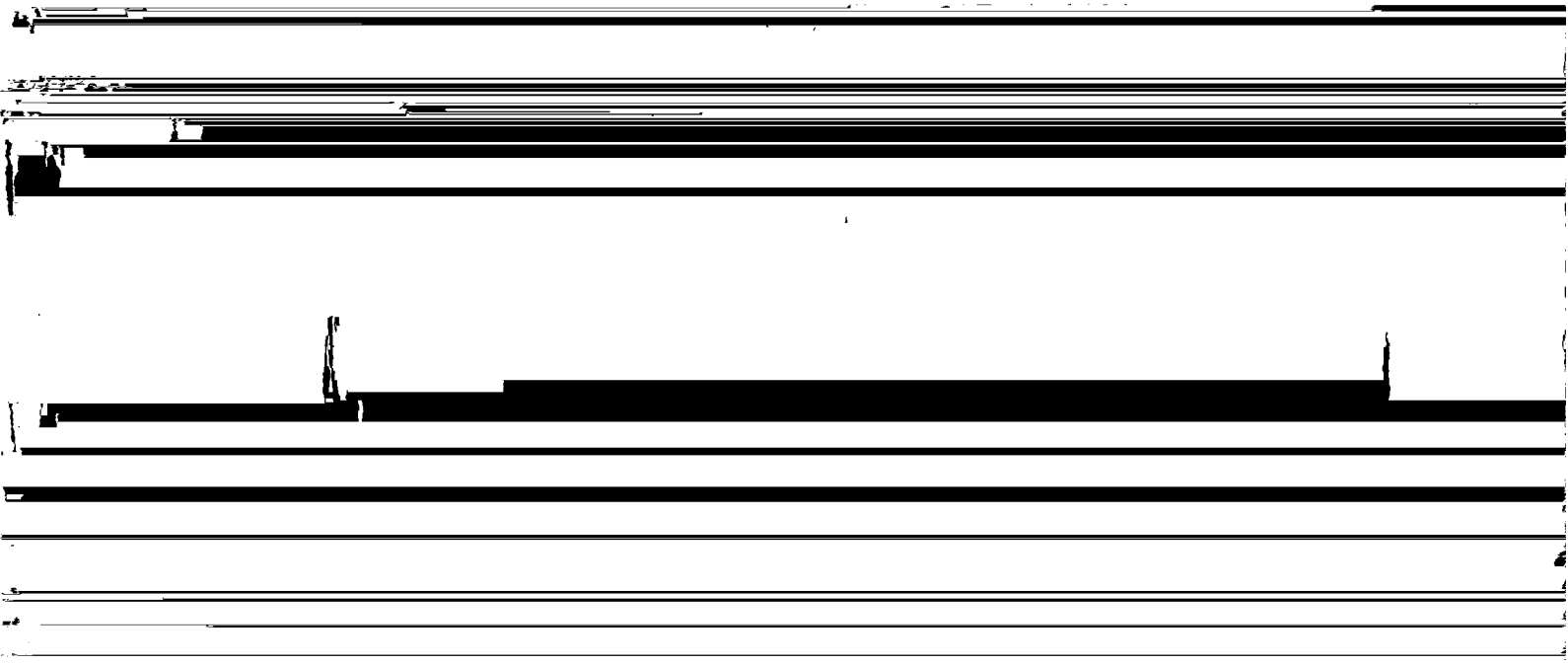
(f) If the monolayer is to be stained later, dry it completely.
(g) If the monolayer is to be stained immediately, rinse it with sterile water and discard the wash.
(h) Add working solution of bisbenzimide or some other suitable DNA staining agent and allow to stand for 10 min.
(i) Remove the stain and rinse the monolayer with sterile water.
(j) Mount each coverslip with a drop of a mixture of equal volumes of glycerol and phosphate–citrate buffer solution at pH 5.5; remove excess mountant from the edge of the coverslip by blotting.
(k) Examine by epifluorescence (330 nm/380 nm excitation filter, LP 440 nm barrier filter) at 100–400× magnification or greater.
(l) Compare the microscopic appearance of the test cultures with that of the negative and positive controls.

The product to be examined passes the test if there is no evidence of the presence of mycoplasmas in the test cultures inoculated with it.

Reagents

Bisbenzimide ($C_{25}H_{27}Cl_3N_6O \cdot 5H_2O$ (M_r 624)) 4-[5-[5-(4-methylpiperazin-1-yl)benzimidazol-2-yl]benzimidazol-2-yl]phenol trihydrochloride pentahydrate.

Bisbenzimide stock solution: To prepare stock solution, dissolve 5 mg



solvent. Store in the dark.

Bisbenzimide working solution: To prepare working solution, dilute 10 µl of bisbenzimide stock solution to 100 ml with phosphate-buffered saline at pH 7.4. Use immediately.

Phosphate–citrate buffer solution, pH 5.5: To prepare buffer, mix 56.85 ml of a 2.84% (28.4 g/l) solution of anhydrous disodium hydrogen phosphate and 43.15 ml of a 2.1% (21 g/l) solution of citric acid."

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Reference

General Requirements for the Sterility of Biological Substances (Requirements for Biological Substances No. 6, revised 1973). In: *WHO Expert Committee on Biological Standardization. Twenty-fifth Report.* Geneva, World Health Organization, 1973, Annex 4 (WHO Technical Report Series, No. 530).