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Test method for bactericidal effect of disinfectant in laboratory

消毒剂实验室杀菌效果检验方法

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Test method for bactericidal effect of disinfectant in laboratory

1 Scope

This standard specifies the terms and definitions, basic requirements, test methods for bactericidal effect of disinfectant in laboratory.

This standard is applicable to the inspection and evaluation of the bactericidal effect of various disinfectants in the laboratory.

2 Normative references

The following documents are essential to the application of this document. For the dated documents, only the versions with the dates indicated are applicable to this document; for the undated documents, only the latest version (including all the amendments) is applicable to this standard.

GB 19489 Laboratories - General requirements for biosafety

3 Terms and definitions

The following terms and definitions apply to this document.

3.1

Disinfectant

Preparations, which are used to kill microorganisms on the transmission medium, to achieve disinfection or sterilization requirements.

3.2

Neutralizer

In the microbial killing test, a reagent, which is used to eliminate the residual disinfectant in the suspension of the test microorganism and the disinfectant and on the surface of the microorganism, so that it loses the inhibitory and killing effect on the microorganism.

3.3

b) For laboratory tests to evaluate disinfectants, the test concentration of the disinfectant shall be the lowest use concentration of the disinfectant, which is specified in the product specification, for a representative disinfection object. Three different action times are set in the test. In principle, the first time is 0.5 times the shortest action time, which is specified in the manual; the second time is the shortest action time; the third time is 1.5 times the shortest action time.

c) For multi-purpose disinfectants, when the microorganisms involved in the disinfection objects are the same, if the concentration used is the same, THEN, select the shortest action time, among various purposes. If the duration of action is the same, select the lowest use concentration for each application. When the low concentration and short action time AND the high concentration and long action time coexist, the former shall prevail. When using high concentration and short action time coexist with low use concentration and long action time, each dose shall be tested.

d) Qualitative test of carrier shall be used for sterilization test. Stainless steel sheet is used as carrier for sterilization of common medical devices. Glass sheet, polytetrafluoroethylene sheet, etc., can be used for special purposes. The sterilization test is carried out, according to the lowest use concentration (intensity) and 0.5 times the shortest action time, which are specified in the product specification. The carrier qualitative test shall be repeated 5 times. The total number of samples shall be no less than 30. A specified number of negative controls and positive controls shall be established for each test.

e) When conducting laboratory tests, the concentration of the organic interfering substance - bovine serum albumin - is 3.0%, for the disinfectant, which is used for unwashed or relatively dirty disinfection objects. The concentration of the organic interfering substance - bovine serum albumin - is 0.3%, for the disinfectant, which is used for cleaned or relatively clean disinfection objects. The organic interfering substance may not be used, for the disinfectant, which is used for the disinfection object that has been strictly cleaned or extremely clean.

4.2.2 Requirements for repeated tests

For the repeatability test, it shall not only increase the number of bacterial slices in the same test, OR make several more samples, BUT shall be carried out in stages and in batches. Necessary equipment and reagents shall be re-prepared or sterilized, to prevent systematic errors.

4.2.3 Evaluation of results

Disinfection products, that meet all the following corresponding conditions, are judged to be qualified in the disinfection effect test results:

a) The qualification test for the effect of removing residual disinfectant is qualified.

5.1.2.1 Preparation of bacterial propagule suspension

5.1.2.1.1 Open the strain tube, by aseptic operation. Use a capillary pipette, to add an appropriate amount of nutrient broth medium. Blow and suck several times, to melt and disperse the strain. Take a test tube, which contains 5.0 mL ~ 10.0 mL of nutrient broth medium. Add dropwise a little strain suspension. Incubate it at 36 °C ± 1 °C for 18 h ~ 24 h. Use the inoculation ring, to take the bacterial suspension of the first generation culture. Streak to inoculate it on the nutrient agar medium plate. Incubate it at 36 °C ± 1 °C for 18 h ~ 24 h. OR take out a bacterial bead, from the strain storage tube. Inoculate it on a plate. Incubate it at 36 °C ± 1 °C for 18 h ~ 24 h. Pick out the typical colonies in the second generation culture above. Inoculate it on a nutrient agar slant. Incubate it at 36 °C ± 1 °C for 18 h ~ 24 h, which is the third generation of culture.

5.1.2.1.2 Take the fresh slant culture, which is obtained by cultivating the 3rd to 8th generation of nutrient agar medium, for 18 h ~ 24 h. Use a 5.0 mL pipette, to draw 3.0 mL ~ 5.0 mL of dilution (usually TPS, acidified water uses normal saline). Add it into the test tube. Repeat blowing and sucking, to wash the bacterial moss. Then, use a 5.0 mL pipette, to transfer the lotion to another sterile test tube. Use an electric mixer, to mix it for 20 s. OR vibrate 80 times on the palm of hand, to suspend the bacteria evenly.

5.1.2.1.3 For the initially prepared bacterial suspension, first use the bacterial concentration turbidimetric method, to roughly measure the bacterial concentration; then use a diluent, to dilute it to the desired concentration.

5.1.2.1.4 The bacterial propagule suspension shall be stored in a refrigerator, at 4 °C for future use. It shall be used in the same day of preparation. It is not allowed for overnight.

5.1.2.1.5 When contamination is suspected, it shall be identified, by methods such as colony morphology, Gram staining, biochemical tests.

5.1.2.2 Preparation of bacterial spore suspension

5.1.2.2.1 Open the strain tube by aseptic operation. Use a capillary pipette, to add an appropriate amount of nutrient broth medium. Blow and suck several times, to melt and disperse the strain. Take a test tube, which contains 5.0 mL ~ 10.0 mL of nutrient broth medium. Add dropwise a little strain suspension. Incubate it at 36 °C ± 1 °C, for 18 h ~ 24 h. Use the inoculation ring, to take the bacterial suspension of the first generation culture. Streak to inoculate it on the nutrient agar medium plate. Incubate it, at 36 °C ± 1 °C for 18 h ~ 24 h. Pick the typical colonies in the above second generation culture. Inoculate it in nutrient broth medium. Incubate it at 36 °C ± 1 °C for 18 h ~ 24 h, which is the third generation culture.

5.1.2.2.2 Use a 10.0 mL pipette, to take 5.0 mL ~ 10.0 mL of the 18 h ~ 24 h nutrient broth culture of the 3rd ~ 5th generation. Inoculate it on the surface of the nutrient agar medium in the Roche bottle. Shake it, to make the bacterial liquid spread fully on the surface of the nutrient agar medium. Then suck out the excess broth culture. Place the

Roche flask, in a constant temperature incubator, at 36 °C ± 1 °C for 5 d ~ 7 d.

5.1.2.2.3 Use an inoculation ring, to take a little bacterial moss and spread it on the glass slide, to stain it, by the improved spore staining method.

The steps of the improved spore staining method are as follows:

a) Use the inoculation ring, to take the bacterial moss and spread it on the glass slide. Let it dry naturally. Then fix the bacteria on the glass slide, by flame heating.

b) Put the smear into the plate. Put two layers of filter paper on the smear. Add dropwise a sufficient amount of 5.00% malachite green aqueous solution. Cover the plate well. Put it under the condition of 54 °C ~ 56 °C. Heat it for 30 min. Take it out. Remove the filter paper. Use tap water to rinse off the residual liquid.

c) Add 0.5% safranine water solution. Stain it for 1 min. Use water to rinse it. Carry out microscopic examination after drying. The spores are green, whilst the cells are red. Under the microscope (oil lens), carry out microscopic examination. When the spore formation rate reaches more than 90%, the follow-up treatment can be carried out. Otherwise, it shall continue to be placed at room temperature for a certain period of time, until the above-mentioned spore formation rate is reached, before the following treatments are performed.

5.1.2.2.4 Add 10.0 mL of sterile distilled water to the Roche bottle. Use a L stick to gently push, to scrape off the bacterial moss. Suck it out. Then add 5.0 mL of sterile distilled water, to rinse the surface of the medium. Suck it out. Collect the bacterial suspension, which is sucked twice, in a sterile conical flask, which contains glass beads. Shake it for 5 min.

5.1.2.2.5 Put the flask in a 45 °C water bath for 24 h, so that the bacteria will self-dissolve and cut off the chain, to disperse into single spores.

5.1.2.2.6 Use sterile cotton or gauze, to filter the spore suspension, to remove the agar clot.

5.1.2.2.7 Put the spore suspension in a sterile centrifuge tube, to centrifuge it at 3000 r/min for 30 min. Discard the supernatant. Add distilled water for blowing and sucking, to resuspend the spores. Repeat this step 3 times.

5.1.2.2.8 Put the washed spore suspension into a flask, which contains an appropriate amount of small glass beads. Place it in a water bath, at 80 °C for 10 minutes (or 60 °C for 30 minutes), to kill the remaining bacterial propagules. After cooling to room temperature, shake well and store in a 4 °C refrigerator for later use. It is valid for half a year.

5.1.2.2.9 When the spore suspension is used, the viable bacteria shall be cultured and counted first.

5.1.3.5 The amount of recovered bacteria per tablet (carrier) shall be 1×10^6 CFU/piece ~ 5×10^6 CFU/piece.

5.1.4 Notes

5.1.4.1 The concentration of the bacterial suspension, which is determined by the turbidimeter, is only used to estimate the dilution of the bacterial suspension, when dripping the bacterial tablet. As the official report of the bacterial concentration of the bacterial suspension or the bacterial count of the bacterial tablet (such as the bacterial count of the bacterial suspension of the positive control group or the bacterial tablet in the sterilization test), it shall be based on the actual measurement result of the viable bacterial culture count. It should not use the estimated value, based on turbidimetric determination.

5.1.4.2 Bacteria drop should not be added too fast, to avoid the accuracy of contamination due to flow.

5.1.4.3 During the drying process of bacterial propagules on the carrier, part of them may die. The initial bacterial concentration should be increased, in order to achieve the required amount of recovered bacteria.

5.1.4.4 When preparing bacterial suspensions and bacterial tablets, strict aseptic operation shall be performed, to prevent contamination of miscellaneous bacteria and affect the results of the sterilization test.

5.1.4.5 When a rubber stopper is used, in the container for storing bacterial liquid, it shall be boiled for 10 min in advance, for desulfurization treatment.

5.1.4.6 The bacterial suspension and bacterial tablet shall be placed in the refrigerator at any time. The storage time at room temperature shall be shortened as much as possible, to reduce the natural death of bacteria.

5.2 Counting technology of viable bacteria culture

5.2.1 Experimental equipment

5.2.1.1 Experimental reagents

Diluent, bacterial culture medium, Tryptone Soy Agar (TSA), Tryptone Soy Broth (TSB), etc. (see Appendix B).

5.2.1.2 Experimental equipment and consumables

Graduated pipette (1.0 mL, 5.0 mL, 10.0 mL), pipette (10 μL, 20 μL, 100 μL, 200 μL, 1 mL, 5 mL) and matching plastic tips, electric mixer, turbidimeter, constant temperature incubator.

5.2.2 Operating procedure

The counting of viable bacteria culture generally uses the pouring method (except for those with special requirements). The operating procedure of pouring method is as follows:

a) The bacterial suspension can be directly cultured and counted. Put the bacterial tablets and small solid samples, directly into a sterile test tube, which contains 5.0 mL of diluent. Cut the sampling end of the cotton swab, into the tube. Use an electric mixer, to mix it for 20 s; OR vigorously vibrate 80 times on the palm of hand, to wash the bacteria, to form a bacterial suspension.

b) Arrange the test tubes on the test tube rack, in groups according to the required number. Add 4.5 mL of diluent to each tube. From left to right, mark each tube, with 10^{-1}, 10^{-2}, 10^{-3}, ..., etc., one by one.

c) Use an electric mixer, to mix the bacterial suspension sample for 20 s, OR vigorously vibrate it 80 times on the palm of hand. Then pipette 0.5 mL into the 10^{-1} tube.

d) Use an electric mixer, to mix the 10^{-1} tube for 20 s, OR vigorously vibrate it 80 times on the palm of hand. Mix well. Then draw out 0.5 mL. Add it into the 10^{-2} tube, and so on, until the last tube. If necessary, it can also be diluted, at a dilution of 1:1 or 1:4.

e) Select a test tube, which has an appropriate dilution (the expected number of colonies per plate should be 15 CFU ~ 300 CFU). Pipette 1.0 mL of the evenly mixed suspension, into a sterile plate. Two plates are inoculated for each dilution. Generally, 2 ~ 3 different dilutions shall be inoculated.

f) Pour the melted culture medium at 40 °C ~ 45 °C into the plate, to which the sample solution has been added, 15 mL ~ 20 mL per plate.

g) Cover the plate. Immediately shake gently to mix. Lay it flat. After the agar is solidified, turn the plate, so that the bottom is up. Place it in a constant temperature incubator, at 36 °C ± 1 °C for culture (the culture temperature for spores of stearothermophilus is 56 °C ± 2 °C; the culture temperature for Aspergillus niger is 30 °C ± 1 °C).

h) Cultivate to the specified time. Count the number of colonies. For field test samples, the colony count shall be observed and recorded daily.

i) When counting colonies, generally observe with the naked eye. Check with a magnifying glass, if necessary. Record the results, based on the dilution of the number of colonies per plate within 15 CFU ~ 300 CFU. When counting the viable bacteria of Aspergillus niger, record the results, based on the dilution of 15 CFU ~ 100 CFU of colonies per plate. For samples with a very small amount of

5.2.4.7 The temperature of the medium during pouring shall not exceed 45 °C, to prevent damage to bacteria or fungi.

5.2.4.8 The pouring and shaking shall be as stable as possible. The medium shall not be spilled, so as to ensure that the bacteria are dispersed evenly, to facilitate counting the colonies.

5.3 Removal method of residual disinfectant (chemical factor)

5.3.1 Principle requirements

5.3.1.1 Residual disinfectants or the effects of disinfectants shall be effectively removed.

5.3.1.2 It is harmless to the test microorganisms AND does not reduce the amount of recovered bacteria.

5.3.1.3 Do not destroy the nutrients of the medium AND do not affect its transparency.

5.3.2 Removal method

5.3.2.1 Dilution neutralization method (neutralizer method)

When the interaction between disinfectant and microorganisms reaches the set time, take sample. Add it to the neutralizer of suitable type and concentration. Neutralize the residual disinfectant quickly, so that it can no longer continuously kill and inhibit microorganisms. The key points of its operation are as follows:

a) Pipette the microbial samples, which have been acted on by the disinfectant, into the certified neutralizer solution, immediately after reaching the specified action time;

b) The concentration and usage of neutralizer used shall be the same as those specified in the identification test results;

c) Immediately mix it well. Pipette sample solution, at the specified time, for subsequent culture testing;

d) The operation, before the sample is inoculated into the medium, shall be carried out within the specified time, so as to prevent the microorganisms from being in contact with the neutralizing agent or the neutralizing product for a long time.

5.3.2.2 Filter rinsing method

Immediately add the microbial samples, that have been subjected to the action of the disinfectant, to an appropriate amount of diluent. Mix evenly (appropriate dilution can reduce the continuous effect of the disinfectant). Pour it into a filter, which is equipped with a microporous membrane. Connect it to a vacuum pump for suction and filtration

(or pressure filtration). Add an appropriate amount of diluent to rinse. Filter it at the same time, to remove residual disinfectant. It is mostly used for disinfection effect tests, where it is difficult to find suitable neutralizers. The key points of its operation are as follows:

a) The microporous membrane and filter shall be used, after sterilization;

b) After the initial filtration, it shall be rinsed by a microbiologically harmless diluent, subject to removal of disinfectant;

c) After rinsing and filtering, take out the microporous filter membrane by aseptic operation; then carry out the subsequent culture test.

5.3.3 Notes

5.3.3.1 The sterile pipette shall be replaced every time the liquid is suctioned, to prevent cross-contamination.

5.3.3.2 The capacity of the straw to be used shall be as close as possible to the amount of liquid to be absorbed. Do not use a large straw, to absorb a small amount of liquid.

5.3.3.3 The test conditions can affect the removal effect of residual disinfectant, so each time a disinfection effect test is carried out, the selected method shall be subjected to an identification test of the removal effect, according to the requirements.

5.4 Neutralizer identification test

5.4.1 Experimental equipment

5.4.1.1 Experimental bacterial suspension and bacterial tablet (see 5.1).

5.4.1.2 Experimental reagents: Diluent, culture medium (see Appendix B).

5.4.1.3 Experimental equipment and consumables: Graduated pipette (1.0 mL, 5.0 mL), plate, constant temperature water bath, electric mixer.

5.4.2 Design principles

5.4.2.1 Through the comprehensive analysis of the test results of each set, it shall be possible to determine whether the neutralizer used has a good neutralizing effect AND has no adverse effect on the recovery and cultivation of the microorganisms used in the test.

5.4.2.2 The concentration of disinfectant, which is used in the test, shall be the highest concentration, which is used in the sterilization test.

5.4.2.3 When the same disinfectant is used for the killing test of multiple

5.5.1.3 Other equipment shall be determined, based on the test microorganism.

5.5.2 Design principles

5.5.2.1 Through the comprehensive analysis of the test results of each group, it shall be possible to determine whether the selected method has a good removal effect on the test disinfectant; it has no adverse effect on the recovery and cultivation of the microorganisms, which are used in the test.

5.5.2.2 The concentration of disinfectant, which is used in the test, shall be the highest concentration, which is used in the sterilization test.

5.5.2.3 When the same disinfectant is to be tested for killing multiple microorganisms, the neutralizing agent used shall be identified and tested, according to the type of microorganism, as follows:

a) Bacterial propagules, which can be tested, in any one of Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa;

b) Bacterial spores, Candida albicans, Aspergillus niger, Mycobacterium shall be identified separately;

c) When the killing test is carried out by other specific microorganisms, the identification test of the neutralizer shall be carried out, by the specific microorganism.

5.5.2.4 In the identification, an appropriate test method shall be selected, according to the design of the killing test. The general suspension identification test results can be used for the carrier test.

5.5.3 Qualification of filter rinse removal method

According to the experimental groups, prepare enough test tubes and plates. Number them in sequence. Use an equal amount of organic interfering substances of suitable concentration, as the test bacterial suspension, to dilute the bacterial suspension to 1×10^2 CFU/mL ~ 5×10^2 CFU/mL. The test was divided into the following 3 groups:

a) Group 1: Pipette 1.0 mL of the test bacteria suspension, into the test tube. Add 4.0 mL of standard hard water. Mix well. Take 1.0 mL and add it to the filter. Then add 50 mL of distilled water for rinsing and filtration. Directly paste the filter membrane, with the bacteria face up, on the plate surface. Place it in a constant temperature incubator, at 36 °C ± 1 °C for 48 hours (fungi and spores are cultured for 72 hours). Count the number of colonies;

b) Group 2: Pipette 0.2 mL of the test bacteria suspension directly into the filter. Then add 150 mL ~ 500 mL of rinse solution to the filter, to do the first rinse and filter treatment. Add 50 mL of distilled water, to do the second rinse and filter

5.6.4.2.3 Put the plate, which contains the disinfectant, in a water bath, at 20 °C ± 1 °C for 5 minutes. Use sterile tweezers, to take 6 pre-prepared bacterial tablets AND put them into the plate, respectively. Immerse them in the disinfectant.

5.6.4.2.4 After the bacteria to be tested interact with the disinfectant for a set time, use sterile tweezers, to take out the bacterial tablets and transfer them to test tubes, which contain 5.0 mL of neutralizer broth, respectively. Use an electric mixer, to mix for 20 s, as the sample of the experimental group.

5.6.4.2.5 Take another plate. Add 20.0 mL of standard hard water instead of disinfectant. Put in 4 bacterial tablets. Let it action for the set time. Take out 2 tablets. Transfer them into test tubes, which contain 5 mL of neutralizer, respectively. The subsequent steps are the same as the above experimental group, as the positive control group sample. Take another 2 tablets, into a test tube, which contains 5.0 mL of neutralizer broth, respectively. Culture and count the viable bacteria, according to 5.2, as the bacterial count control group sample.

5.6.4.2.6 All test samples are cultured in a constant temperature incubator, at 36 °C ± 1 °C. The bacterial propagules are cultured for 48 h, to observe the final results. The bacterial spores are cultured for 7 days, to observe the final results.

5.6.4.2.7 The test is repeated 5 times, to calculate the amount of viable bacteria (CFU/tablet) in each group.

5.6.5 Quantitative killing test of membrane filtration suspension

5.6.5.1 The preparation of bacterial suspension and the quantitative killing test are the same as those in 5.6.3.1, 5.6.3.2, 5.6.3.3; the pore size of the filter membrane is 0.45 μm.

5.6.5.2 The bacteria to be tested and the disinfectant (respectively placed in a water bath at 20° C ± 1 °C for 5 minutes in advance) interact for each set time. Respectively pipette 1.0 mL of the mixture of test bacteria and disinfectant, into the filter for filtration. Then add 150 mL ~ 500 mL of rinsing solution, for the first rinsing and filtration treatment. Then add 50 mL of distilled water, for the second rinsing and filtration treatment. Paste the filter membrane, with the bacterial side up, on the surface of the plate. Place it in a constant temperature incubator at 36 °C ± 1 °C, to culture it to the specified time.

5.6.5.3 Pipette 0.5 mL of the test bacteria suspension into the test tube. Add 0.5 mL of organic interfering substances. Add 4.0 mL of standard hard water. Mix well. After 10-fold serial dilution, take 1.0 mL of appropriate dilution. Add it to the filter. Then add 50 mL of distilled water, for rinsing and filtration treatment. Paste the filter membrane, with the bacterial side up, on the surface of the plate. Place it in a constant temperature incubator, at 36 °C ± 1 °C, to culture to specified time. Count the number of colonies, as a positive control.

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