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PROCEDURE FOR TESTING THE ANTIBACTERIAL EFFECT OF THE AIR FILTER AFTER CONTAMINATION THROUGH A BACTERIAL BIOAEROSOL

ICS:

CCMC will prepare and attach the official title page.

Contents

Page

Foreword

This CEN Workshop Agreement (CWA XXXX:YYYY) has been developed in accordance with the CEN-CENELEC Guide 29 "CEN/CENELEC Workshop Agreements – A rapid way to standardization" and with the relevant provisions of CEN/CENELEC Internal Regulations - Part 2. It was approved by the Workshop CEN/WS "Evaluating Antimicrobial Coatings: From Air Filtration Efficiency to Antiviral Mechanism and Ecotoxicology", the secretariat of which is held by UNE (Spanish Association for Standardization) consisting of representatives of interested parties on 2025-07-29, the constitution of which was supported by CEN following the public call for participation made on 2025-06-16. However, this CEN Workshop Agreement does not necessarily include all relevant stakeholders.

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Introduction

Indoor air quality (IAQ) is a critical factor in ensuring public health, particularly in environments like hospitals, transportation systems, and workplaces. Antimicrobial air filtration technologies have attracted significant attention as a solution to reduce microbial transmission through HVAC (Heating, Ventilation, and Air Conditioning) or HEPA (High-Efficiency Particulate Air) systems.

Despite the growing availability of filters with antimicrobial treatments, there is currently no standards for evaluating their antibacterial performance under realistic airborne exposure. Most existing test methods focus on surface or water-based applications and do not reflect the aerosol contamination typically encountered in HVAC or HEPA systems.

This CEN Workshop Agreement outlines a methodology to evaluate antibacterial performance under realistic HVAC or HEPA bioaerosol conditions. During the process, a bioaerosol containing a high concentration of a specific bacterial strain is nebulized onto the filter, which is placed inside a filter holder for a defined period. The method employs both qualitative and quantitative analysis to assess bacterial survival on filter surfaces. The qualitative assessment consists of visually evaluating bacterial growth on agar plates in contact with contaminated filter specimens after incubation. The quantitative approach entails counting the colony-forming units (CFU) from a nutrient broth in which the contaminated filter has been immersed and subsequently incubated. Both approaches include the comparison with a control air filter without antimicrobial agents tested in the same way.

This document has been based on the knowledge generated in the EU-funded research project NANOBLOC, which received funding from the European Union's Horizon Europe research and innovation programme under grant agreement No. 101057597.

1 Scope

This document defines a standardized method for assessing the antibacterial effectiveness of air filtration media after exposure to a bacterial bioaerosol. The procedure includes the controlled generation and delivery of a bacterial aerosol, its contact with the test filter surface, and the subsequent evaluation of bacterial viability using both qualitative and quantitative approaches.

This method is applicable to various types of air filters, including but not limited to HEPA filters, HVAC filters, coated filters, and filters treated with antimicrobial agents.. It establish validation criteria for controls, inoculum quality, and test conditions to ensure reproducibility and comparability of results across laboratories and applications.

This procedure is specifically designed for air filters treated with antibacterial agents to confer antimicrobial properties. Untreated specimens of the same type and production batch shall be used as control specimens to assess the relative antibacterial performance under identical test conditions.

The method is intended for use in research, quality control, and product development. It may also be used to facilitate conformity assessment procedures and regulatory compliance, where relevant and applicable.

2 Normative references

There are no normative references in this document.

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply:

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at http://www.iso.org/obp/

— IEC Electropedia: available at http://www.electropedia.org/

3.1

Antibacterial activity

The ability of a material or substance to inhibit or reduce the growth of bacteria, either on its surface or in surrounding media.

3.2

HEPA filter (High-Efficiency Particulate Air filter)

A type of air filter capable of removing at least 99.95% of airborne particles of 0.3 microns in diameter, according to EN 1822 classification.

3.3

HVAC (Heating, Ventilation and Air Conditioning systems)

A type of environmental control system designed to regulate indoor temperature, humidity, and air quality through integrated heating, ventilation, and air conditioning components.

3.4

Filters treated with antimicrobial agents

An air filter that has been modified through any process intended to incorporate one or more antibacterial agents in order to confer antimicrobial properties.

3.5

Untreated specimen

A test specimen that does not contain any antimicrobial agent. It serves as a baseline to compare the performance of treated specimens.

3.6

Bioaerosol

A suspension of airborne particles that contains or is derived from living organisms, including bacteria, viruses, fungi, or their fragments. In this context, it refers specifically to a bacterial aerosol used for contamination testing.

3.7

McFarland standard

A turbidity standard used to estimate the concentration of microbial colonies in suspension. A 5 McFarland standard corresponds to approximately 1.5×10^9 CFU/mL.

3.8

CFU (Colony Forming Unit)

A unit used to estimate the number of viable bacteria or fungal cells in a specimen. One CFU is typically assumed to arise from a single organism capable of forming a colony under specific conditions.

3.9

Qualitative analysis

A visual or observational assessment used to determine the presence or absence of bacterial growth, typically on a nutrient agar surface in contact with a test material.

3.10

Quantitative analysis

A measurement-based evaluation of antibacterial activity, typically involving serial dilutions, plating on agar, and counting of CFUs.

3.11

Vortex mixing

A laboratory method used to resuspend cells or dislodge surface-bound bacteria by rapid agitation of a liquid in a tube.

4 Materials

4.1 Bacterial Strain

For the purpose of evaluating the antibacterial effectiveness of air filtration media, it is recommended to perform the test using both a Gram-positive and a Gram-negative bacterial species. This approach enables the assessment of the antimicrobial performance of the treated filter against a broader spectrum of airborne bacteria**.**

The following well-characterised, non-pathogenic reference strains (biosafety level 1 or 2) are commonly used and suggested for this method:

– *Staphylococcus epidermidis* (e.g., ATCC 14990) – Gram-positive
–*Bacillus subtilis* (e.g. ATCC 33234) – Gram-positive
– *Escherichia coli* (e.g., ATCC 8739) – Gram-negative
– *Pseudomonas aeruginosa* (e.g., ATCC 15442) – Gram-negative

Alternative strains may be used provided they are of equivalent biosafety classification and are appropriately justified in the test report.

4.2 Air Filters

The test specimen shall consist of air filters systems or other air filtration media intended for antibacterial performance evaluation. Filters shall be pre-conditioned in accordance with the manufacturer's instructions or the specific test protocol. The dimensions, materials, and filtration classification of each specimen shall be clearly documented.

For each test series, six specimens shall be prepared per analysis type, as follows:

– **Qualitative analysis**: Three treated and three untreated specimens of the same type and production batch shall be cut to match the full area of the filter holder used in the bioaerosol generation system.

– **Quantitative analysis**: Three treated and three untreated specimens shall be cut to a standardized rectangular size of 2.0 cm × 0.5 cm, suitable for immersion in broth and ensuring reproducibility of CFU enumeration.

All specimens shall be handled under aseptic conditions through the preparation and testing process. Untreated control specimens are required to establish the baseline for both qualitative and quantitative analyses.

4.3 Culture Media

The culture medium specified for use in this method shall be suitable for the cultivation and recovery of the selected bacterial strains. The medium may be obtained from commercial suppliers, provided that it is prepared and used in accordance with the manufacturer's instructions.

The type and composition of the culture medium shall be documented in the test report. Any deviations or substitutions shall be justified and validated to ensure compatibility with the test organisms and reproducibility of results.

The composition and preparation of the recommended culture media are detailed in **Annex A**.

5 Equipment and Apparatus

5.1 Bacterial Bioaerosol Generator

A device capable of producing a consistent and controlled bacterial aerosol from a liquid bacterial suspension. The generator shall ensure uniform droplet size distribution and stable output throughout the exposure period. Suitable examples include Collison nebulizers or equivalent systems that have been validated for microbiological aerosol generation.

5.2 Filter Holder

A sealed and sterile chamber shall be employed to securely house the air filter during bioaerosol exposure. The filter holder shall allow uniform exposure of the filter surface to the aerosol stream while preventing leakage or contamination. It shall ensure laminar flow conditions and be compatible with downstream air collection devices.

5.3 Compressed Air Source

A pressure-regulated compressed air supply shall be connected to the bioaerosol generator. The system shall deliver a constant airflow (e.g., 1 bar), and include sterile filtration to avoid introducing external contamination into the aerosol stream.

5.4 Biological Safety Cabinet

All procedures involving the handling of bacterial cultures, including specimen preparation, contamination, and manipulation of exposed filters, shall be performed under a certified laminar flow hood (Class II). This is required to maintain aseptic conditions and ensure operator safety.

5.5 Incubator

An incubator capable of maintaining a stable temperature of 35 ± 2 °C shall be used for all culture and incubation steps. The incubator shall support aerobic conditions and be validated for uniform heat distribution.

5.6 Sonicator

A sonicator shall be employed during the quantitative analysis phase to dislodge adherent bacteria from the surface of the filter specimens. The device shall provide consistent ultrasonic agitation and be compatible with standard laboratory tubes.

5.7 Optical Density Reader

A spectrophotometer or McFarland densitometer shall be used to verify the turbidity of the bacterial suspension prior to aerosol generation. The suspension shall be standardized to 5 ± 1 McFarland units to ensure consistency in bacterial load across test specimens.A spectrophotometer or McFarland densitometer shall be used to verify the turbidity of the bacterial suspension prior to aerosol generation. The suspension shall be standardized to (5 ± 1) McFarland units to ensure consistency in bacterial load across test specimens.

5.8 Spreading Tools and Accessories

The following tools shall be used to ensure accurate and aseptic handling of bacterial suspensions during the test procedure:

- **Sterile spreaders** (e.g. Drigalski spatulas or disposable plastic spreaders): Used to evenly distribute bacterial suspensions across the surface of agar plates for colony enumeration.
- **Sterile disposable pipette tips and micropipettes**: Required for the precise transfer and dilution of bacterial suspensions, ensuring aseptic technique and reproducibility.
- **Sterile test tubes or vials**: Utilized for the preparation, incubation, and handling of bacterial suspensions during the quantitative test.

6 Preparation

6.1 Preparation of Bacterial Suspension for Bioaerosol Generation

A bacterial suspension shall be prepared in 20 mL of Brain Heart Infusion (BHI) Broth, adjusted to a turbidity equivalent to 5 ± 1 McFarland standard, corresponding to approximately 1.5×10^9 CFU/mL. This suspension shall be used to test a complete specimen set, consisting of six untreated and six treated specimens. For each additional sets of specimens, a fresh bacterial suspension shall be prepared to ensure consistency and reproducibility of results.

6.2 Preparation of Test Specimens (Air Filters)

Test filters shall be handled under aseptic conditions in a certified laminar flow cabinet to minimize environmental contamination. All components, including filter holders and handling tools, shall be sterilized or disinfected prior to use.

- **Qualitative analysis**: three untreated and three treated filter specimens shall be cut to match the dimensions of the filter holder used in the aerosol test system (e.g., compatible with the TOPAS ATM 220 setup). The treated side of the test filter shall be oriented to face the direction of the incoming airflow during exposure.
- **Quantitative analysis:** three untreated and three treated rectangular specimens measuring 2.0 cm × 0.5 cm shall be prepared for bacterial recovery and colony enumeration procedures.

Table 1 provides a summary of the number and dimensions of test specimens required for both qualitative and quantitative antibacterial analyses using a single bacterial strain. This configuration shall be repeated for each bacterial strain included in the test protocol.

Table 1: Summary of Specimen Types and Required Materials

Test type	Number of specimens	Filter type and dimensions	Culture media	Notes
Qualitative analysis	6 total (3 treated + 3 untreated specimens)	Full-size or cut to fit filter holder	Mueller-Hinton Agar or Nutrient Agar	The treated side must face airflow; used for contact with agar surface
Quantitative Analysis	6 total (3 treated + 3 untreated specimens)	2.0 cm × 0.5 cm	Mueller-Hinton Broth and saline solution	Standardised size for immersion in broth and CFU enumeration

7 Test Procedure

7.1 General

This section describes the procedures for contaminating air filter specimens with a bacterial bioaerosol and assessing their antibacterial performance using both qualitative and quantitative methods. Antibacterial tests shall be performed in accordance with a standardized method based on NCCLS M7-A6 (1), with the necessary adaptations for the specific case addressed in this document.

7.2 Contamination procedure

7.2.1 Objective

To uniformly contaminate the surface of filter specimens with a bacterial aerosol under controlled conditions, simulating realistic air conditioning systems.

7.2.2 Procedure

- The following steps shall be followed. Connect the bacterial bioaerosol generator (e.g. TOPAS ATM 220) to a calibrated compressed air source regulated at 1 bar.
- Load the prepared bacterial suspension into the generator in accordance with the manufacturer's instructions. Preliminary Evaluation (Test Validity Criterion). To verify the viability and consistency of aerosol deposition, expose a sterile agar plate (Mueller-Hinton agar or Nutrient agar, depending on the bacterial strain used) to the aerosol stream for 15 minutes and 30 minutes. Incubate the plates at (35 ± 2) °C for (24 ± 2) hours and perform colony-forming unit (CFU) enumeration to confirm viable deposition and uniformity.
- Mount each filter specimen into a sterile filter holder with the treated side facing the airflow direction.
- Expose each filter individually to the bacterial aerosol for 15 and 30 minutes by operating the generator under controlled conditions.
- Perform each contamination step separately to maintain test repeatability and avoid cross-contamination between specimens.

Table 2 summarises the operating conditions of the contamination test

Table 2: Summary of the operating conditions of the contamination test

Parameter	Specification	Purpose
Bacterial suspension concentration	5 ± 1 (≈ 1.5×10^9 CFU/mL)	Ensures consistent bacterial load for aerosol generation
Aerosol Exposure Pressure	1 bar	Simulates HVAC conditions
Exposure time per specimen	15 and 30 min	Validates aerosol deposition over time and assesses the time-dependent antibacterial effect

Filter orientation	Treated side facing airflow	Ensures direct exposure of the antimicrobial surface
Incubation Condition	35 ± 2 °C; 24 ± 2 hours	Ensures optimal bacterial growth under standardised exposure time for all specimens

7.2.3 Controls

Three untreated filter specimens shall be included in each test series as negative controls. These control specimens are required for comparative analysis and to establish baseline bacterial deposition levels.

7.3 Qualitative Analysis Test (Observation of Bacteria Growth)

7.3.1 Objective

The objective of this test is to assess the antibacterial activity of the filter surface by visual inspection of bacterial growth in direct contact with the test specimen after incubation on nutrient agar.

7.3.2 Procedure

- The following steps shall be followed. Using sterile forceps, carefully place the contaminated filter specimen onto a sterile Mueller-Hinton Agar or Nutrient Agar plate, ensuring full contact between the contaminated specimen surface and the agar.
- Incubate the plate at 35 ± 2 °C for 24 ± 2 hours under aerobic conditions.
- After incubation, visually examine the contact area between the filter and the agar surface for bacterial growth.

7.3.3 Evaluation

The following check points shall be followed.

- Control filters (untreated) are expected to show dense and confluent bacterial growth in direct contact with the agar.
- Treated filters are expected to show reduced or absent bacterial colonies in the contact area, indicating antibacterial activity.
- The number and density of colonies at the contact zone shall be qualitatively compared between treated and control specimens.
- No inhibition zone is expected; antibacterial activity is inferred only by the reduction or absence of colonies in the contact zone.

7.3.4 Documentation

Photographic evidence of the contact areas is recommended to support qualitative assessment and facilitate comparative analysis.

7.4 Quantitative Analysis Test (Colony-Forming Unit Count)

7.4.1 Objective

The objective of this test is to determine the antibacterial effectiveness of the test filter by counting viable bacteria in liquid culture and those adherent to the filter surface following exposure to a bacterial bioaerosol.

7.4.2 Procedure

The following steps shall be followed.

a) Immersion in Broth

Each exposed filter specimen shall be transferred into a sterile test tube containing Mueller-Hinton broth. The tubes shall be incubated at a temperature of (35 ± 2) °C for (24 ± 2) hours under aerobic conditions.

b) Turbidity Assessment (optional) Broth turbidity may be visually compared against the McFarland scale to obtain a preliminary indication of bacterial growth. This step is optional and intended for qualitative reference only.

c) Serial Dilution and Plating

- **Serial dilution**: Tenfold serial dilutions of the incubated broth shall be performed to obtain a range suitable for plating and enumeration.
- **Plating**: Aliquots (e.g. 100 µL) from each dilution shall be plated onto Blood Agar or Nutrient Agar plates using either the pour plate or spread plate method.
- **Incubation**: The inoculated plates shall be incubated at a temperature of (35–37) °C for a duration of (18–24) hours.

d) Colony Counting and Calculation

- **Colony Counting:** CFUs shall be counted on plates containing between 30 and 300 colonies, which represents the statistically valid range for enumeration.
  CFU counts shall be reported as the number of colonies multiplied by the corresponding dilution factor.

$$CFU = number\ of\ colonies\ x\ diluition\ factor$$

- **Calculation:** The percentage reduction of bacterial colonies on treated specimens compared to untreated controls shall then be calculated to evaluate antimicrobial efficacy of the filter material.

$$\%\ reduction = \left(\frac{CFU_{\ untreated} - CFU_{\ treated}}{CFU_{\ untreated}}\right) x\ 100$$

7.5 Surface-Adherent Bacteria Recovery

7.5.1 Objective

The objective of this procedure is to quantify bacteria that remained adherent to the filter surface after broth immersion , thereby providing an additional measure of antibacterial effectiveness.

7.5.2 Procedure

The following steps shall be followed.

- Remove the filter from the broth and transfer it into a sterile tube containing 3 mL of sterile saline.
- Vortex vigorously for 5 minutes at room temperature to dislodge surface-bound bacteria.
- Perform serial dilutions of the saline suspension and plate as described in Section 7.3.
- Incubate and count CFUs to assess surface bacterial load recovered from the filter surface

A schematic representation of the main steps of the antibacterial test is provided in the flow chart below (Figure 1).

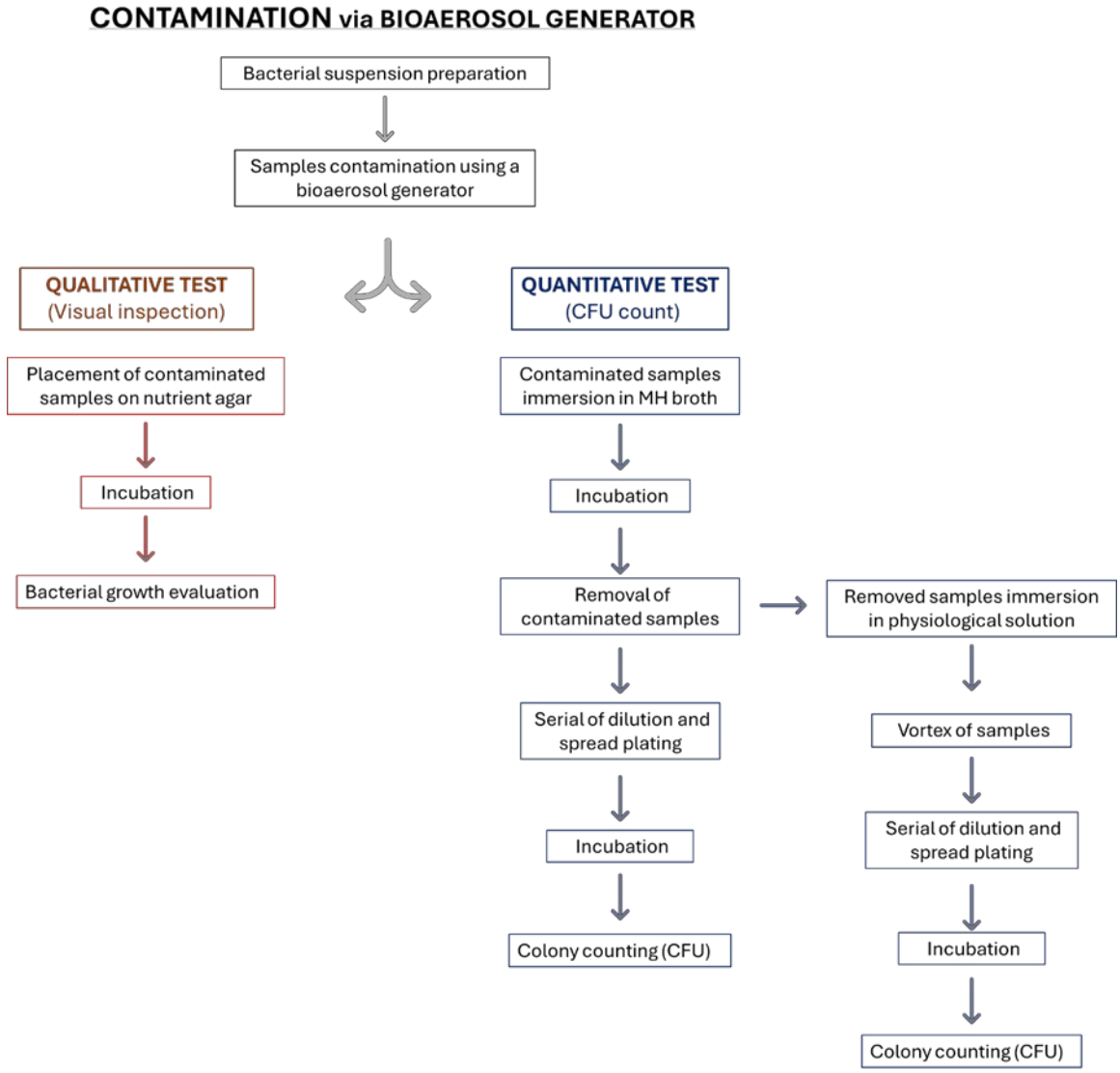

Figure 1: Schematic representation of the antibacterial test procedure.

8 Criteria for test validity

8.1 General

To ensure the validity and reliability of the antibacterial test results, all of the following conditions shall be met.

8.2 Control Validity

- A Mueller-Hinton agar or Nutrient agar plate exposed to the bacterial aerosol shall exhibit dense and uniform bacterial growth after incubation, confirming the effectiveness of the aerosolisation process.
- The same bacterial suspension shall be used for each qualitative and quantitative test set (i.e. three treated and three untreated specimens). A fresh suspension shall be prepared for each additional set.
- All tests shall be conducted under identical environmental and procedural conditions to ensure consistency and comparability across specimens.
- The untreated specimens (negative control) shall show substantial bacterial growth, both qualitatively and quantitatively, confirming the viability of the bacterial suspension and the adequacy of test conditions.

The blank control (sterility check, using broth and agar without bacterial inoculation) shall show no bacterial growth (or only minimal background contamination), verifying the absence of procedural contamination**.**

8.3 Bacterial Suspension Quality

- The initial bacterial suspension used for aerosol generation shall have a turbidity corresponding to 5 ± 1 McFarland standard, equivalent to approximately 1.5×10^9 CFU/mL.
- The bacterial culture used for bioaerosol generation shall be incubated for 18–24 hours at 35 ± 2 °C under aerobic conditions to ensure optimal viability.

8.4 Test Conditions

- Incubation after exposure shall be conducted at 35 ± 2 °C for 24 ± 2 hours.
- The aerosol generation parameters, including exposure time and air pressure (e.g., 1 bar), shall remain constant and be properly recorded.

8.5 CFU Measurement Criteria

- The quantitative test shall yield at least one dilution with colony counts in the statistically acceptable range of 30 to 300 CFU per plate.

If one or more of these criteria are not met, the test is deemed invalid, and the procedure shall be repeated.

In Table 3, the criteria for the validity of the preliminary test and qualitative and quantitative evaluations are reported.

Table 3: Test validity criteria

Test type	Validity criteria
Preliminary test	MHA or NA plates must show dense bacterial growth
Qualitative test	The untreated filter specimens (control) must show dense bacterial growth
Quantitative test	CFU Count Valid Range: 30–300 colonies per plate

9 Result Interpretation

9.1 General

The antibacterial effect of the test specimens shall be evaluated by comparing the results of the treated filters with those of the untreated control filters Both qualitative and quantitative criteria shall be applied to determine the presence and extent of antibacterial activity.

9.2 Qualitative Analysis – Interpretation of Antibacterial Effect

Qualitative antibacterial activity shall be assessed by visually inspecting the agar plate after incubation for signs of bacterial growth in and around the contact area with the test specimen.

The following categories shall be used for interpretation:

- **No antibacterial effect**:
  Bacterial colonies are present in direct contact with the test specimen and also in the surrounding area. The entire surface of the specimen is typically covered with confluent or dense growth.
- **Moderate antibacterial effect**:
  Colonies are observed primarily in the area surrounding the specimen, with reduced or no growth in direct contact with the surface.
  This category also includes cases where:
  - The number of colonies on the specimen is markedly reduced compared to the control
  - Growth is mainly concentrated at the edges of the filter, with the central area showing sparse or no colonies.
- **Clear antibacterial effect**:
  No bacterial colonies are detected either in contact with the test specimen or in the adjacent area.

In all cases, qualitative observations shall include:

- Presence or absence of colonies
- Distribution and density of growth (e.g., sparse, moderate, confluent)

Control filters (untreated) are expected to show confluent or dense bacterial growth in direct contact with the agar, confirming the absence of antibacterial activity. These observations shall be documented photographically and included in the final report.

9.3 Quantitative Analysis – Interpretation of Antibacterial Effect

Quantitative antibacterial activity shall be evaluated by calculating the percentage reduction of CFU in treated specimens compared to untreated controls (as described in Section 7.4). The interpretation shall be based on the following thresholds:

- **No antibacterial effect**: 0% ≤ % reduction < 25%
- **Moderate antibacterial effect**: 25% ≤ % reduction ≤ 75%
- **Clear antibacterial effect**: reduction > 75%

Quantitative results shall be expressed in:

- Absolute CFU/mL
- % reduction compared to the control

Colony Count Validation Criteria:

- Only plates yielding 30–300 colonies per dilution shall be considered statistically valid.
- Results falling outside this range shall be excluded from analysis or require repetition of the test.

Final results shall be reported in comparison to those obtained for untreated specimens, and both the planktonic bacterial load and surface-adherent bacteria shall be considered, where applicable.

10 Expression of Results

10.1 General

Test results shall be expressed in both qualitative and quantitative terms to ensure a comprehensive evaluation of antibacterial performance.

10.2 Qualitative Results

Results obtained from the agar-contact analysis shall be reported as:

- **Qualitative classification**:
  - No antibacterial effect
  - Moderate antibacterial effect
  - Clear antibacterial effect
- **Evaluation method:**
  Based on visual inspection of colony presence in the contact area between the filter specimen and the agar surface.
- **Documentation:**

Photographic records of the contact areas are recommended to support the qualitative assessment and facilitate comparative analysis.

10.3 Quantitative Results

Results from the colony-forming unit (CFU) count shall be reported as follows:

- Colony-forming units per milliliter (CFU/mL)
- Percentage reduction (%) in CFU count compared to the negative control

All values shall be derived from plates within the valid range (30–300 CFU) and supported by replicate measurements. When applicable, quantitative data may be presented in tabular or graphical form (e.g., bar plots of CFU/mL) to facilitate interpretation and comparison.

11 Test Report

A complete test report shall include the following elements:

- **Test identification**
  - Test method reference
  - Date of execution
  - Name of testing laboratory
- **Specimen information**
  - Filter type and treatment (e.g., antimicrobial coating)
  - Manufacturer or supplier
  - Batch/lot number
  - Specimen preparation details
- **Bacterial strain used**
  - Species and strain number (e.g., *Staphylococcus epidermidis*, ATCC 14990)
  - Source of the strain
- **Test conditions**
  - Bacterial concentration (McFarland and CFU/mL)
  - Aerosol exposure parameters (e.g., time, pressure)
  - Incubation time and temperature
- **Control validation results**
  - Negative control (growth confirmation)
  - Blank control (sterility confirmation)
- **Test results**
  - Qualitative results and classification
  - Inhibition zone diameter (if applicable)
  - Quantitative results: CFU/mL, % reduction, log reduction
  - Observations on both planktonic and adherent bacteria (if tested)
- **Interpretation and conclusion**
  - Statement of antibacterial performance based on test outcomes
  - Notes on any deviations or anomalies (if present)

12 Repeatability and Reproducibility

12.1 General

To ensure the reliability of the antibacterial test method, both repeatability and reproducibility shall be assessed and documented.

12.2 Repeatability

Repeatability refers to the consistency of results when the test is performed under identical conditions. The following requirements apply:

- The test shall be performed on three treated filter specimens and three untreated control specimens per bacterial strain for each qualitative and quantitative test. This setup allows for statistical analysis, including calculation of standard deviation, and ensures repeatability and reliability of the results.
- The results shall fall within an acceptable standard deviation, which should be specified based on experimental data or internal validation criteria.
- Deviations greater than the established threshold shall be investigated and documented, including potential sources of error or variability.

12.3 Reproducibility

Reproducibility refers to the consistency of results when the test is replicated under varying conditions. The following requirements apply:

- The method shall be validated through at least two independent repetitions, preferably conducted by different operators or laboratories.
- Differences in equipment, batch numbers, or environmental factors shall be documented to assess robustness.

12.4 Documentation of Variability

All sources of variation shall be recorded, including:

- Operator identity
- Equipment and instrument model or serial number
- Filter batch or lot number
- Environmental parameters (e.g., temperature, humidity)

Statistical data such as standard deviation, coefficient of variation (CV), or confidence intervals should be reported when available.

These criteria support the method's qualification for inter-laboratory comparison, standardization, and regulatory adoption.

Bibliography

(1) NCCLS M7-A6, Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that grow Aerobically, Approved standard 6th ed. NCCLS, Villanova, PA, USA 2003s

Annex A
(informative)

Composition and preparation of media

A.1 General

The media are available on the market and used for cell culture.

A.2 Brain Heart Infusion (BHI)

Brain Heart Infusion (BHI) is a highly nutritious medium used for cultivating fastidious and non-fastidious microorganisms, including aerobic and anaerobic bacteria, yeasts, and molds. Dissolve 12.5 g of calf brain infusion (dehydrated), 5.0 g of beef heart infusion (dehydrated), 10.0 g of proteose peptone 2.0 g of dextrose (glucose), 5.0 g of sodium chloride, 2.5 g of disodium phosphate in 1 L of distilled water. Heat gently, if necessary, to dissolve the medium completely. Adjust the pH to 7.4 ± 0.2 at 25 °C. Dispense into suitable bottles or tubes. Sterilize by autoclaving at 121°C (15 psi) for 15 minutes. Cool and store at 2–8°C until use.

A.3 Cation Adjusted Mueller-Hinton Broth (CAMHB) with TES

Cation Adjusted Mueller-Hinton Broth (CAMHB) with TES is a specialized medium formulated according to standardized guidelines for antimicrobial susceptibility testing, particularly for broth dilution methods. Dissolve 17.5 g of Casein acid hydrolysate, 3.0 of beef extract and 1.5 g of starch (with cation adjustments and TES buffer) in 1 L of distilled water, using gentle heat if needed. Adjust the pH to 7.3 ± 0.2 at 25 °C. Sterilize by autoclaving at 115–121 °C for 10–15 minutes. Store the sterile medium at 2–8 °C, protected from light.

A.4 Blood agar (BA)

Dissolve 10.0 g of peptone, 10.0 g of tryptose (or tryptic soy), 5.0 g of sodium chloride and 15.0 of agar in 1 L of distilled water. Heat with stirring until fully dissolved; bring to boil briefly to ensure agar is melted completely. Sterilize by autoclaving at 115–121 °C for15 minutes and cool the medium to approximately 45–50°C. Under aseptic conditions, slowly add 50 mL of sterile defibrinated sheep blood to the cooled agar base, gently mixing to avoid bubbles and hemolysis. Pour into sterile petri dishes on a level, horizontal surface to achieve uniform depth, and allow to solidify at room temperature. Store plates at 2–8°C until use.

A.5 Mueller-Hinton Agar (MHA)

Mueller-Hinton Agar (MHA) Plate supports growth of a wide range of non fastidious bacteria. Dissolve 2.0 g of beef extract, 17.5 g of acid hydrolysate of casein, 1.5 g of starch, 17.0 g of agar in 1 L of distilled water. Heat with frequent agitation and bring to a boil for about one minute to fully dissolve the powder. Adjust the pH to 7.3 ± 0.1 at 25 °C. Sterilize by autoclaving at 115–121 °C for 10–15 minutes and cool the medium to approximately 45–50°C. Pour into sterile petri dishes on a level, horizontal surface to achieve uniform depth, and allow to solidify at room temperature. Store plates at 2–8°C until use.

A.6 Nutrient Agar (NA)

Nutrient Agar (NA) Plate is a general-purpose medium suitable for cultivation of many non-fastidious microorganisms. Dissolve 5.0 g of peptone, 3.0 g of beef extract, 5.0 g of sodium chloride and 15.0 g of agar in 1 L of distilled water. Heat with frequent agitation and bring to a boil for about one minute to fully dissolve the powder. Adjust the pH to 7.0 ± 0.2 at 25 °C. Sterilize by autoclaving at 115–121 °C for 10–15 minutes and cool the medium to approximately 45–50°C. Pour into sterile petri dishes on a level, horizontal surface to achieve uniform depth, and allow to solidify at room temperature. Store plates at 2–8°C until use.

A.6 Saline Solution A 0.9% sodium chloride (NaCl) sterile physiological saline solution shall be used for serial dilutions and for recovering surface-adherent bacteria from test specimens. The solution must be sterile and stored under aseptic conditions.