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Evaluation of bactericidal activity of a non-porous antimicrobial surface used in a dry environment

Évaluation de l'activité bactéricide d'une surface antimicrobienne non poreuse utilisée dans un environnement sec

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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO document should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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This document was prepared by Technical Committee ISO/TC 330, *Surfaces with biocidal and antimicrobial properties*.

Any feedback or questions on this document should be directed to the user’s national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

The surfaces in our environment can constitute significant reservoirs of bacteria and a risk to health that is essential to control.

In healthcare establishments, healthcare-associated infections (HAI) are a major cause of mortality and disability the world over. HAI affects 5 % to 15 % of hospital patients in developed countries and can affect 9 % to 37 % of patients admitted to intensive care units. Each year, about 1 in 25 U.S. hospital patients is diagnosed with at least one infection related to hospital care alone. At least five million HAI are estimated to occur in acute care hospitals in Europe, causing 135 000 deaths per year and approximately 25 million additional days spent in hospital, representing a financial cost of €13 billion to €24 billion.

In public transport, the risks of contamination between travellers are extremely high due to the frequency and number of contacts points. In establishments receiving the public, all common areas and collective spaces constitute a risk of the spread of pathogens microorganisms.

In the agri-food sector, microbial hazards are the highest risk amongst those that can impact consumer health, above malnutrition and chemical contaminants. In addition to the impact on health, the consequences of a case of contamination can be disastrous for the image and sustainability of a company. For instance:

— an estimated 600 million – almost 1 in 10 people in the world – fall ill after eating contaminated food and 420 000 die every year, resulting in the loss of 33 million healthy life years [disability-adjusted life years];

— US$110 billion is lost each year in productivity and medical expenses resulting from unsafe food in low- and middle-income countries;

— children under 5 years of age carry 40 % of the foodborne disease burden, with 125 000 deaths every year;

— diarrhoeal diseases are the most common illnesses resulting from the consumption of contaminated food, causing 550 million people to fall ill and 230 000 deaths every year.

The drive to control microbial risks extends to many other sectors (transport, pharmaceuticals, aeronautics, cosmetics, the phytosanitary sector, services, etc.), and even to the entire industrial manufacturing sector.

Pathogenic agents can be transmitted in a variety of ways, such as via food and water, via air (aerosols), via body contact with infected person and via surfaces contaminated by body secretions and fomites. Healthcare-associated infections, or infections acquired in healthcare settings are the most frequent adverse event in healthcare delivery worldwide. Hundreds of millions of patients are affected by healthcare-associated infections worldwide each year, leading to significant mortality and financial losses for health systems. Of every 100 hospitalized patients at any given time, 7 in developed and 10 in developing countries will acquire at least one healthcare-associated infection. Such infections annually account for 37 000 attributable deaths in Europe and potentially many more that can be related, and they account for 99 000 deaths in the USA. Amongst the available tools for reducing microbial risks, surfaces with biocidal properties can help to control cross-contaminations when used in combination with standard cleaning and disinfecting practice.

This document was written to address the need to demonstrate the biocidal efficacy of a surface under ambient conditions close to the conditions found in the field. The method described in this document simulates the contamination of a surface by a microdroplet. For example, this pathway of contamination is representative of a surface contamination following a sneeze (microdroplet post sneeze might have a mobility of several meters). The temperatures and humidity required for the test were defined in relation to conditions that are representative of the atmosphere in the healthcare facilities.

Numerous non-porous surfaces claim to perform a bactericidal function:

— surfaces of materials that have been treated with or include a biocidal product in order to give the material bactericidal properties, either temporarily or permanently.

— surfaces of materials, such as certain metals, that claim to have intrinsic bactericidal properties.

The method prescribes the representative basic strains to be tested. Additional strains may be tested, depending on the intended use. For a given use, further experiments including in use condition (soiling substances, ageing, adaption of environmental condition) are needed for demonstrating bactericidal activity of the tested surface as claimed.

This method will be revised in the near future to include interfering substances and to adapt strains/efficacy/contact time specifications to the needs of different sectors, in addition to the medical area.

Surfaces with bactericidal properties supplement, but do not replace, the regular use of surface-treatment products, such as detergents, detergent-disinfectants and surface disinfectants which must be demonstrated to be compatible with maintaining the surface bactericidal activity.

Evaluation of bactericidal activity of a non-porous antimicrobial surface used in a dry environment

WARNING — Persons using this document shall have technical microbiological knowledge.

1 Scope

This document specifies the test conditions and the levels of activity to determine the bactericidal activity of non-porous surfaces used in a dry environment. It defines a protocol to validate the bactericidal character of a surface and to measure its performance. It is not intended to be used to substantiate cleaning or disinfecting properties.

This document is applicable to surfaces claiming to have an activity against vegetative bacteria. The obligatory test conditions are defined in this document. It does not apply to porous surfaces.

It does not refer to methods for testing the toxicological and ecotoxicological properties of the surfaces. This document is used to measure bactericidal action, not bacteriostatic activity of a surface.

2 Normative reference

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

EN 12353, *Chemical disinfectants and antiseptics — Preservation of test organisms used for the determination of bactericidal (including Legionella), mycobactericidal, sporicidal, fungicidal and virucidal (including bacteriophages) activity*

3 Terms and definitions

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

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

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

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

3.1
bactericidal activity
capability of a surface to produce a reduction in the number of viable bacterial cells of representative *test organisms* (3.6) under defined conditions without soil load, nor ageing test nor test conditions adaptation to a specific sector.

Note 1 to entry: Note to entry: At the moment this document does not include soiling or ageing condition.

3.2
bacteriostatic activity
capability of a surface to inhibit the growth of viable bacterial cells of representative *test organisms* (3.6) under defined conditions

Note 1 to entry: The term "bacteriostatic activity" cannot be used for claims according to this document.

3.3
additional condition
test conditions that are optional and not obligatory, that can be used for additional claims of *bactericidal activity* (3.1) regarding a surface

3.4
cleaning
all operations that achieve a level of cleanliness, appearance, comfort and hygiene

Note 1 to entry: Such operations use, to varying degrees, the following combined factors: chemical action, mechanical action, temperature, duration of action

3.5
neutralizer
chemical agent or formulation that suppresses the residual microbicidal activity of a product or active substance from the surface to be tested for a specific test but does not inactivate or inhibit the *test organism* (3.6)

3.6
test organism
strain of a microorganism selected to evaluate the antimicrobial activity of a surface for a standardized test

3.7
bactericidal surface
surface that irreversibly kill vegetative bacteria under defined conditions

Note 1 to entry: The adjective “bactericidal” corresponds to the noun “bactericide”.

3.8
reference surface
surface without any *bactericidal activity* (3.1) or properties, that is used to evaluate the quantity of culturable bacteria present at the moment when the bactericidal activity of the *test surface* (3.9) is evaluated

3.9
test surface
surface claiming *bactericidal activity* (3.1)

Note 1 to entry: This method is suitable for testing any type of non-porous surfaces (such as metals, plastic, glass, coated surfaces, etc.) as long as recovery of bacteria from the test surface shall not lose more than 1 log at T=0.

Note 2 to entry: Very hydrophobic surfaces, for which a drying time greater than 10 min (see 6.2.2) is necessary, cannot be tested according this method.

3.10
ambient light
light corresponding to a maximum value of 2 000 lux

Note 1 to entry: Any specific light or light spectrum (e.g. strong light, UV, etc.) is not considered as ambient.

3.11
porous surface
surface permeable to water, air, or other fluids

4 Apparatus, reagents and materials

4.1 Test organisms

Bactericidal activity shall be evaluated using the following four strains:

— *Pseudomonas aeruginosa* ATCC 15442 = CIP 103-467;

— *Staphylococcus aureus* ATCC 6538 = CIP 4.83;

— *Enterococcus hirae* ATCC 10541 = CIP 5855;

— *Escherichia coli* ATCC 10536 = CIP 54127.

The reference strain numbers in other culture collections shall be in accordance with the strains specified in Annex A.

The activity data can be completed with other strains using the experimental design described in this document, varying the conditions to meet the needs of the intended -practice application(s). If additional strains are used, they shall be incubated under optimum growth conditions (temperature, time, atmosphere) to be recorded in the test report.

Their suitability for supplying the inoculum and controls with a sufficient concentration shall be verified. If these additional test strains are not classified at a culture collection centre, their identification characteristics shall be stated. In addition, they shall be held by the testing laboratory or national culture collection under a reference for a five-year period.

NOTE ISO/TC 330 is currently adapting the method in terms of strains/efficacy levels/contact times/interfering substances according to different sectors' needs and to include not only the medical area.

4.2 Culture media and reagents

The reagents shall be of analytical grade and/or appropriate for microbiological purposes. They shall be free from substances that are toxic or inhibitory to the test organisms.

4.2.1 Water, which shall be free from any substances that are toxic or that inhibit the bacteria. It shall be freshly glass-distilled water or water for injection or possibly deionized or demineralized water.

Sterilize in the autoclave (4.4.1).

NOTE If the water is sterilized during the sterilization of the reagents, this is not necessary.

4.2.2 Microbial suspension diluents

Tryptone-salt solution:

Tryptone, pancreatic digest of casein	1,0 g
Sodium chloride	8,5 g
Water (see 4.2.1)	1,000 ml

Sterilize in the autoclave (see 4.4.1). After sterilization, the pH of the medium shall be equivalent to 7,0 ± 0,2 when measured at 20 °C.

4.2.3 Liquid for bacteria recovery and for membrane rinsing

4.2.3.1 Composition

Tryptone, pancreatic digest of casein	1,0 g

Sodium chloride	8,5 g
Polysorbate 80	5,0 g
Water (see 4.2.1)	1 000 ml

4.2.3.2 Preparation

a) Dissolve the sodium chloride and tryptone in the water (see 4.2.2). Add the Polysorbate 80, mix and complete up to 1 000 ml with water.

b) Distribute into smaller adapted flasks. Sterilize in the autoclave.

c) If necessary, add a neutralizer to the recovery liquid.

The use of a validated neutralizer of the respective antimicrobial active substance is compulsory to warrant a correct test time for bacteria recovery and for membrane rinsing (see 4.2.3). The functionality of the respective neutralizer shall be validated beforehand. The neutralizer:

— shall be validated for the tested surface, as per 6.2.4 and it shall be sterile;

— shall be mentioned in the test report.

Examples of neutralizers are given in Annex D.

4.2.4 Agar for bacteria maintenance and counting [tryptone soya agar (TSA)]

Use agar to preserve the bacterial strains and count the viable bacteria.

Tryptone, pancreatic digest of casein	15,0 g
Soya peptone, papaic digest of soybean meal	5,0 g
Sodium chloride	5,0 g
Agar	15,0 g
Water (see 4.2.1)	1 000 ml

Sterilize in the autoclave. After sterilization, the pH of the medium shall be equivalent to 7,2 ± 0,2 when measured at 20 °C. Pre pored plate TSA are acceptable if they meet the composition and pH value described in 4.2.4.

4.3 Reference and test surfaces

4.3.1 General

The surfaces shall be used only once.

4.3.2 Reference surfaces

4.3.2.1 Description

Inox 304 stainless steel, disk 2 cm in diameter, of which both sides have a grade 2B finish. The surfaces shall be flat.

For the testing of surface coated, if the coated surface without antimicrobial agent (the test surface) exists, it can be used as a reference surface similar to inox and therefore can be used for log reduction calculation.

The reference surface used during the test shall be specified in the report.

4.3.2.2 Cleaning — Disinfection/sterilization

The reference surfaces shall be cleaned and disinfected before use.

If a specific preparation protocol is applied to the test surface (see examples in Annex C), the same protocol should be applied to the reference surface (see 4.3.3.2). Otherwise, the protocol in example 1 of Annex C should be used.

No residual action or changes of the antimicrobial properties shall result from disinfection/cleaning procedure on the reference surface.

The reference surface treatment used during the test shall be specified in the report.

4.3.3 Test surfaces

4.3.3.1 Identification and production

The characteristics (e.g. dimensions, thickness, etc.) and references of the finished product shall be defined and specified in the final test report.

If the test surface is treated with a bactericide, the nature of the active substance(s) shall be specified to the lab for safety and security reasons, and indicated in the final test report. For coated surfaces with unknown porosity (e.g. paint), recovery of the microorganisms shall be checked by the laboratory, comparing the coated surface with the antimicrobial agent and the reference surface (C0 vs T0) or the coated surface with and without the antimicrobial agent, to ensure the accuracy of bacteria reduction evaluation. Recovery of bacteria from the test surface shall not lose more than 1 log at T=0 compared to the reference one.

The test surface shall be produced from the materials intended for the finished product claiming bactericidal activity, and according to the same steps of the final production process. If it is impossible to perform the steps on the same equipment, it is permitted to use a representative simulation of these steps.

The test surface shall have flat surfaces and measure between 12 mm and 25 ± 2 mm on the sides or in diameter. If it is impossible to cut the test surface into squares or disks of this size, other sizes and shapes can be used. In this case, the actual dimensions used will be stated in the test report. Any changes made to the protocol (recovery volume, etc.) due to the dimensions of the tested test surface shall be specified in the test report.

The uniformity of each test surface shall be visually inspected. Test surfaces with anomalies on the surface or edges shall be discarded (e.g. corrosion/rust, slivers, deep grooves or ridges, etc.).

4.3.3.2 Cleaning — Disinfection/sterilization

All the test surfaces shall be at least rinsed with sterile distilled water before performing the tests.

If necessary, disinfection or sterilization can be performed according to the nature of the surface, indicated by the manufacturer and detailed in the test report. The manufacturer shall propose/validate the complete treatment. If necessary, preliminary tests shall be performed to define the appropriate treatment to avoid impacting the efficacy of the tested surfaces. These tests can result in the methodology described in 4.3.2.2 being adapted with identical treatment for the reference surface and the test surface.

No residual action or changes of the antimicrobial properties shall result from disinfection/cleaning procedure on the reference and tested surface.

Annex C contains examples of surface preparation protocols.

The test surface treatment used during the test shall be specified in the report.

4.4 Apparatus and materials

Sterilize all glassware and parts of the apparatus that will come into contact with the culture media and reagents or the sample, except those which are supplied sterile, by one of the following methods:

— in the autoclave (see 4.4.1), by keeping them at between 121 °C and 124 °C for at least 15 min;

— in the hot air oven (see 4.4.1), by keeping them at 180 °C for at least 30 min, at 170 °C for at least 1 h or at 160 °C for at least 2 h.

Usual microbiological laboratory equipment, and in particular:

4.4.1 Apparatus for sterilization

— For moist heat sterilization, an autoclave capable of being maintained at between 121 °C and 124 °C for at least 15 min.

— For dry heat sterilization, a hot air oven capable of being maintained at between 180 °C and 185 °C for at least 30 min, at between 170 °C and 175 °C for at least 1 h, or at between 160 °C and 165 °C for at least 2 h.

4.4.2 Water baths. Thermostat-controlled bath at 45 °C ± 1 °C.

4.4.3 Incubators

— Incubators capable of being maintained in the range of 35 °C to 38 ± 1 °C.

— Thermostat-controlled incubators at the defined test temperatures (excluding ambient temperature).

4.4.4 pH-meter, accurate to ±0,1 pH units at 20 °C ± 1 °C

For measuring the pH of the agar media, a puncture electrode or a flat membrane electrode shall be used.

4.4.5 Calibrated thermometer or temperature probe accurate to ±1 °C.

4.4.6 Calibrated relative humidity meter accurate to ±2 % RH .

4.4.7 Light measuring apparatus.

4.4.8 Stopwatch.

4.4.9 Mechanical or electromechanical shaker.

4.4.10 Refrigerator, temperature-controllable to between 2 °C and 8 °C.

4.4.11 Graduated pipettes of nominal capacities of 10 ml, 2 ml, 1 ml and 0,1 ml. Calibrated automatic pipettes may be used, in particular for small volumes (inoculum deposit).

4.4.12 Petri dishes, 90 mm to 100 mm in diameter. 55 mm Petri dishes can be used for the membranes.

4.4.13 Glass beads (diameter: 3 mm to 5 mm).

4.4.14 Analytical balance of suitable operating range.

4.4.15 Spectrophotometer fitted with a monochromator.

4.4.16 Vacuum pump (max. flow rate 3,8 m^3/h, final vacuum < 7,5 kPa).

4.4.17 0,45 µm pore-size **sterile filter membranes** (diameter 47 mm to 50 mm) in cellulose ester or any other suitable material.

4.4.18 Microscope, fitted with x40 and x100 immersion lenses (purity checks after colouring: strain visual control).

4.4.19 Forceps.

4.4.20 Sterile test tubes in neutral glass, 18 mm or 20 mm in diameter.

4.4.21 Recipients, flasks or vials of adequate capacity for the intended use.

4.4.22 Class II biological safety enclosure (that can also be used in the stage to dry the inocula).

4.4.23 Freezer, with a temperature equal to or lower than – 70 °C.

4.4.24 Membrane filtration apparatus, built in a material compatible with the filtrate substances.

The apparatus shall be equipped with a funnel with a capacity of at least 50 ml. It shall be suitable for use with 47 mm to 50 mm-diameter, 0,45 µm pore-size filters. The vacuum source used shall give an even filtration flow rate. In order to obtain a uniform distribution of the microorganisms over the membrane and prevent filtration that lasts too long, the targeted filter speed is 100 ml of rinsing liquid in less than 40 seconds.

4.4.25 Climatic/Seal chamber or box capable of controlling temperature and relative humidity.

5 Preparation of the test organism suspensions

5.1 Stock cultures of the test bacteria

The test organisms and their stock cultures shall be prepared and kept in accordance with the requirements of EN 12353.

5.2 Working culture of the test bacteria

To prepare the working culture of the test organisms, prepare a subculture from the stock culture (see 5.1) by streaking onto TSA slopes (slants) or plates (4.2.4, before incubating them (see 4.4.3.). After incubating for 18 h to 24 h, take the first subculture and prepare a second one in the same way, and incubate for 18 h to 24 h. The second subculture can be used to prepare a third one in the same manner.

The second and/or third subcultures are the working cultures.

If it is not possible to prepare the second subculture on a particular day, a 48 h ± 2 h subculture may be used for subsequent subculturing, provided that the subculture has been kept in the incubator over the 48 h ± 2 h period. In this case, prepare another 24-hour subculture before running the test. Do not prepare a fourth subculture.

For additional strains (see 4.1), any departure from this bacterial culture method or test suspension preparation method shall be recorded in the test report, with the reasons substantiating this departure.

5.3 Test suspensions

Take 10 ml of diluent (see 4.2.2) and place in a 100 ml flask or container with 5 g of sterile glass beads (see 4.4.13). Take loopfuls of working culture (see 5.2) and transfer it into the diluent. The cells shall be suspended in the diluent by immersing the loop in the diluent and by rubbing it against the wall of the vessel to dislodge the cells. Shake the vessel for 3 min using a mechanical shaker (see 4.4.9). Aspirate the suspension, leaving the glass beads at the bottom, and transfer it to another vial. Adjust the number of cells of the suspension to a value between 1,5 10^8 CFU/ml and 5,0 10^8 CFU/ml[1)] using the diluent, estimating the number by appropriate means (i.e. spectrophotometry). The suspension shall be used within 2 h of preparation.

For Gram negative bacteria, use the top part of the inoculum.

The test laboratory is responsible for the precision of the titres obtained in the preliminary tests.

5.4 Quantifying of bacterial test suspensions

Dilute the adjusted bacterial suspensions to 10^{-6} (serial dilutions) and 10^{-7} using the diluent (see 4.2.2). Mix the suspension (see 4.4.9).

Take a duplicate sample of 1,0 ml of each dilution and inoculate using the pour plate technique. Use a pipette to introduce each 1,0 ml sample into separate Petri dishes (see 4.4.12) and add 15 ml to 20 ml of molten TSA agar (see 4.2.4).

The spread plate technique can also be used. In this case, use 100 µl of bacterial suspensions 10^{-5} and 10^{-6} (serial dilutions).

Incubate the dishes in the range of 35 °C to 38 °C ± 1 °C for 24 h ± 2 h. Discard any dishes which are not countable (for any reason). Incubate the dishes for a further 24 h ± 2 h. Do not repeat the count on the dishes for which it is not possible to count the colonies. Count the remaining dishes again.

Determine the highest colony count for each sample.

See 6.2.2 i) for the incubation and counting.

5.5 Calculation of the weighted mean bacteria concentration in the test suspensions

a) Discard all the dishes for which counting is impossible (for any reason). Determine the total number of CFUs per dish. Do not repeat the count on the dishes for which the colonies are not clearly separate. Count the remaining dishes again after 48 h. If the number has increased, only use the higher number for the subsequent calculations.

b) Only the dishes with a number of colonies between 14 and 330 for the bacteria are used to calculate the result.

   Calculate the weighted mean values of the test suspension by using Formula (1):

$$X = \frac{c}{(n_1 + 0{,}1 n_2)} \times d \quad (1)$$

1) CFU = Colony forming unit

where

X is initial suspension concentration in CFU/ml (refer to 7.2);

c is the sum total of the values of *Viable* cultivable bacteria (*Vc*) considered;

n_1 is the number of values of *Vc* considered in the lowest dilution;

n_2 is the number of values of *Vc* considered in the highest dilution;

d is the dilution level corresponding to the lowest dilution.

Calculation examples following the use of the pour plate method:

EXAMPLE 1:

V_c for n_1: 215 and 230

V_c for n_2: 25 and 30

c = 215 + 230 + 25 + 30 = 500

n_1 the number of values of *Vc* considered in the lowest dilution, i.e. 10^{-6}; 2

n_2 the number of values of *Vc* considered in the highest dilution, i.e. 10^{-7}; 2

$$X=\frac{500}{(2,2)}\times 10^{6}$$

So

X= 2,27. 10^8 CFU/ml (8,36 log CFU/ml)

EXAMPLE 2:

V_c for n_1: 312 and 342 (>330: not included)

V_c for n_2: 32 and 35

c = 312 + 32 + 35 = 379

n_1 the number of values of *Vc* considered in the lowest dilution, i.e. 10^{-6} ; 1

n_2 the number of values of *Vc* considered in the highest dilution, i.e. 10^{-7} ; 2

$$X=\frac{379}{(1,2)}\times 10^{6}$$

X= 3,16. 10^8 CFU/ml (8,50 log CFU/ml)

6 Evaluation method of the bactericidal activity of a non-porous surface and its efficacy

6.1 Obligatory experimental conditions

The defined experimental conditions to determine the bactericidal activity of a non-porous surface are as follows:

a) test temperature shall be 20 ± 1 °C; additional temperatures can be chosen and shall be reported in the test report;

b) relative humidity shall be between 30 % and 65 % (additional levels of relative humidity can be chosen and shall be reported in the test report);

c) ambient light;

d) contact time, t (minutes): minimum 60 min ± 10 s to maximum 120 min ± 10 s;

e) strains: they shall match those specified in 4.1 and annex A.

Additional conditions (light, strains, contact time) can be tested using the described protocol, provided that all the test validation criteria are met and reported in the test report.

Test shall be performed using a climatic/seal chamber or box (see 4.4.25).

6.2 Test procedure

6.2.1 Sampling

Each test to evaluate bactericidal activity shall be performed on at least three test surfaces and reference surfaces. Ultimately, eight test surfaces (and 6 reference surfaces for 6.2.3) are the minimum for performing the test (test surfaces: 3 for T_0 and 3 for T_{xh} and 2 for Vn; reference surfaces: 3 for C0 and 3 for Cxh). If 1H and 2H be both tested in the same run, 11 samples for the test surfaces are needed (3 for T_0, 3 for T1h, 3 for T_{2h} and 2 for Vn).

Using more than three test surfaces per test can help to reduce variability and, therefore, the value of the standard deviation used in the calculation of the log reduction.

6.2.2 Test (T_0 and T_{xh})

This subclause details the test to evaluate the number of viable bacteria remaining on the test surface (4.3.3).

The data corresponding to time 0 (preferably < 3 min not exceeding 10 min for drying) are identified as T_0. The data corresponding to time 1 h are identified as T_{1h}. . The data corresponding to the contact time x are identified as T_{xh} (example: T_{1h} or T_{2h}).

Perform the following for each test strain.

a) Put the test surfaces (4.3.3) in a sterile Petri dish and make sure that the dish is horizontal.

b) Inoculate each test surface with 1 µl of the final test suspension (5.3) using a calibrated pipette or a calibrated loop.

NOTE Hydrophobic surfaces are treated in the same way.

c) Spread the inoculum to about 3 mm from the edge of each test surface (over a surface of about 10 mm × 10 mm), using the tip of the pipette. Guidance for inoculum deposit is given in Annex E.

d) Leave to dry in a laminar flow hood and record the drying time T_0, which shall be <3 min to 10 min maximum.

e) Record the contact time, which starts after drying is complete.

f) Make a record of the temperature and relative humidity in the laboratory during the contact time. The test surfaces shall remain horizontal and in the ambient conditions, with the cover on the Petri dish.

g) After a contact time of 0 (end of the drying time preferably <3 min to 10 min) to 1 h to 2 h, aseptically transfer each surface into a separate recipient containing 10 ml of recovery liquid (4.2.3) and sufficient glass beads (for example 5 g) to support the surface. The samples should be placed with the inoculated surface downwards in contact with the beads. Manually stir the recipient for at least 1 min. Force shall be used so that the beads move freely under the test surface. Any additional contact time can be done as long as the inoculum remain enough concentrated on the control surface (4 log is the minimum).

h) After stirring (a neutralization time of 5 min ± 10 s can be necessary for certain surfaces), prepare a series of decimal solutions, from 10^{-1} to 10^{-4}, of the mixture in the diluent (4.2.2). Take a double sample of 1,0 ml of the initial mixture and of each of the dilutions, and inoculate the dishes using the pour-plate technique.

i) Then filter the remaining liquid, i.e. about 7 ml, in the sterile filtration apparatus. Filter and rinse the membrane three times using 50 ml of recovery liquid (4.2.3) previously poured into the flask and stirred with the surface. Then, transfer the membrane onto the solidified agar medium for counting.

j) Retrieve the test surface, allow the liquid to flow off, and rinse with 10 ml of water (4.2.1). Transfer the surface into a Petri dish containing 10 ml of solidified TSA agar, placing the inoculated surface side down onto the agar. Add 10 ml of molten TSA agar, cooled to 45 °C.

k) Incubate the dishes at 35 °C to 38 °C for 24 h ± 2 h. Discard any dishes which are not countable (for any reason) and record the counts for the rest. Incubate the dishes for a further 24 h ± 2 h. Do not repeat the count for colonies on dishes which no longer show well-separated colonies. Count them again on the remaining dishes. Only the dishes with a number of colonies between 15 and 300 are used to calculate the result. A deviation of 10 % is permitted. Therefore, the limits for the bacteria are 14 and 330.

6.2.3 Reference surfaces (C_0 and C_{xh})

For each test strain, treat at least 3 reference surfaces according to the instructions in 6.2.2, at times 0 and xh. For reference surfaces, use at least dilutions 10^{-2}, 10^{-3} and 10^{-4} for CFU counts.

The data corresponding to time 0 are identified as C_0. The data corresponding to the contact time x are identified as C_{xh}.

Any additional contact time can be done as long as the inoculum remain enough concentrated on the control surface (4 log is the minimum).

If a difference is detected between T_0 and C_0 then an activity is identified during the wet phase and shall be mentioned in the test report.

6.2.4 Validation (V_n)

For each strain, it is necessary to validate the experimental conditions for each tested surface, in particular regarding the neutralization of the antibacterial residual activity. To this end:

a) Put two test surfaces (4.3.3) in a sterile Petri dish and make sure that the dish is horizontal.

b) Put 1 µl of tryptone salt diluent (4.2.2) on each sample using a calibrated pipette or a calibrated loop.

c) Spread the diluent to about 3 mm from the edge of each sample (over a surface of about 10 mm × 10 mm) using the loop or the tip of the pipette. Leave to dry in a laminar flow hood and confirm the drying time, which shall be preferably ≤ 3 min not exceeding 10 min.

d) Make a record of the start of the contact between the inoculum and the sample.

e) Make a record of the temperature and relative humidity in the laboratory during the contact time. The samples shall remain horizontal and in the ambient conditions, with the cover on the Petri dish. Apply the defined light conditions.

f) After a contact time of 0 (end of drying time), aseptically transfer each surface into a separate recipient and add 10 ml of recovery liquid (4.2.3) and 5 g of glass beads. Add 1 µl of the test suspension at 10^8 CFU/ml. Stir the recipient under the same conditions as in the test. After stirring (a neutralization time of 5 min ± 10 s can be necessary for certain surfaces), prepare a series of decimal solutions, from 10^{-1} to 10^{-4}, of the mixture in the diluent (4.2.2). Take a double sample of 1,0 ml of the initial mixture and of each of the dilutions and inoculate the dishes using the pour-plate technique. Spread-plate technique can be used as an alternative to pour-plating.

Perform step k) described in the test given in 6.2.2.

The data are identified V_n. If V_n does not meet the defined criteria, add a neutralizer to the recovery solution and/or improve the detachment procedure for each strain.

7 Results

7.1 Determination of the number of viable bacteria

Determine the number of colony-forming units (S) remaining on the test surface.

Discard all the dishes for which counting is impossible (for any reason). Determine the total number of CFUs per dish. Do not repeat the count on the dishes for which the colonies are not clearly separate. Count the remaining dishes again. If the number has increased, only use the higher number for the subsequent calculations.

Dishes with a number of colonies between 15 and 300, or between 15 and 150 for counting by filtration (F), are considered in the calculation of the result. A deviation of 10 % is permitted. Therefore, the limits are respectively 14 and 330 and 14 and 165.

Calculate T_0, T_{1h}, C_0, C_{1h} and V_n and the CFU log number that can be retrieved from the reference surface and the test surface using the Formula (2) (weighted mean value):

$$T0, Txh, C0, Cxh, Vn = \frac{c}{(n_1 + 0{,}1 n_2)} \times 10 \times d \quad (2)$$

where

c the sum total of the values of Vc (viable cultivable bacteria) considered in CFUs;

n_1 the number of values of Vc considered in the lowest dilution;

n_2 the number of values of Vc considered in the highest dilution;

d is the dilution level corresponding to the lowest dilution.
10 is the dilution factor corresponding to the recovery in 10 mL of recovery liquid

EXAMPLE 1 If one or two of the duplicate values of Vc are either below the lower limit, or above the upper limit, express the results as “lower than” or “greater than”.

If all the *Vc* values of the counts by pour plate are < 14, take into account the usable counts (CFU < 165; including the values < 14) by filtration (F) and by pour plate of the surface (S) in the agar, according to Formula (3):

$$\frac{F\times10}{7}+S \quad (3)$$

If the *Vc* values of the sample 10^0 (considered as c, where n_0 is the number of values taken into account) and of the count by filtration are > 14, take these values into account according to Formula (4):

$$\left[\frac{F\times10}{7}+\frac{c}{n0}\right]/1{,}1+S \quad (4)$$

EXAMPLE 2

F= 180 CFU; c= 22 CFU and 26 CFU (n_0=2); S=0 CFU

= [(180x10/7) + (22+26)/2]/1,1 + 0 CFU

= (257 + 24)/1,1 + 0 CFU

= 255 CFU

In the test, when the number of CFUs counted on each dish, including counts by filtration and counts from the surface included in the agar, is 0, consider the final value T_{xh} as equal to 0.

When rounding the figures, if the last figure is greater than or equal to 5, increase the preceding figure by one unit. If the last figure is below 5, do not change the preceding figure.

Note values T_0 , T_{xh}, C_0 and C_{xh} for the test and reference surfaces, respectively. Calculate the mean log for each type of surface samples (3 or more) ± standard deviation (SD) for each contact time.

7.2 Verification of the methodology

X (the initial suspension) shall be between 1,5 10^8 and 5,10^8 CFU/ml.

N is the number of theoretical CFUs deposited on the surface (between 1,5 10^5 and 5 10^5 CFU)

V_n shall differ from N by < 2 log – the difference between the two surfaces shall not be > 0,3 log. If V_n does not meet the defined criteria, add a neutralizer to the recovery solution. See Formula (5):

$$(C_{0max} - C_{0min})/(C_{0mean}) \leq 0{,}3 \quad (5)$$

If the difference is > 0,3 log, increase the number of surfaces.

For the reference surfaces, calculate the values of C_0 and C_{xh} – calculate the mean log ± SD. The value of the mean log - SD shall be greater than or equal to 3 (expected log reduction for the active surfaces).

S is less than 100 CFU/ml for active surfaces. If this is not the case, insufficient bacteria have been recovered.

Check of the counts obtained by the weighted mean: the quotient is neither less than 5 nor more than 15.

7.3 Expression of results

7.3.1 Reduction

For each test organism, make a record of the mean log CFU per reference and test surface ± SD at 1 h to 2 h.

Calculate the log reduction using Formula (6):

$$R = C_{xh} - T_{xh} \qquad (6)$$

where R is the reduction expressed as a base ten logarithm.

The results shall be rounded to two significant figures after the coma. When rounding the figures, if the last figure is greater than or equal to 5, increase the preceding figure by one unit.

NOTE The log reduction at time 0 (after the drying time) can be produced for reference purposes (rapid bactericidal activity).

7.3.2 Conclusion: Bactericidal activity of non-porous surfaces

The bactericidal activity of the tested surfaces is defined when the criteria in 7.2 are met and a reduction of minimum 2 log or more is demonstrated under the required test conditions: 60 min to 120 min contact, between 18 °C and 25 °C, at a relative humidity between 30 % and 65 %, when the test organisms are *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterococcus hirae* and *Escherichia coli*. Tested surface can claim to be a bactericidal surface when the above-mentioned criteria are met.

In this way, bactericidal activity under other conditions (temperature, humidity, contact time) can be specifically claimed and clearly labelled when a reduction of viability equal to or greater than 2 log is demonstrated with the defined test organisms (additional strains).

This evaluation method of the bactericidal activity of a non-porous surface and its efficacy can be used to validate the continuation of the activity over time of the bactericidal surface.

If the test is not performed under normal test conditions as defined in 6.1 a) to e), the test report shall clearly state the deviations and the reason for the deviations.

8 Test report

The test report shall include at least the following information:

a) a reference to this document, i.e. ISO 7581:2023;

b) identification of the test laboratory;

c) identification of the sample:

   1) test surface: product name and description of the characteristics (the materials used for the samples and controls (if different from those recommended by this document), and the size, shape and thickness).

   2) reference surface (inox and/or coated surface without active substance): batch number or production reference;

   3) the manufacturer;

   4) date of receipt;

   5) storage conditions;

   6) active substance(s) and concentration(s) (for surfaces with added active substances - optional).

d) the experimental conditions:

   1) analysis period;

   2) number of reference and test surfaces

   3) treatment (cleaning - disinfection) of the reference and test surfaces;
   4) Drying time of the reference and test surfaces
   5) contact time;
   6) test temperature(s);
   7) relative humidity during the test;
   8) identification of the bacterial strains used (obligatory and additional strains);
   9) incubation temperature.
   10) additional conditions

e) procedure: all the details of the tests shall be provided:
   1) validation tests (Vn);
   2) evaluation of the bactericidal activity (T_0, T_{xh}, C_0, C_{xh})

f) conclusion;

g) identified place, date and signature(s).
   1) Details of any departures from this document and the alternative methods used, where appropriate.

   NOTE An example test report is provided in Annex B.

Annex A
(normative)

Corresponding obligatory reference strains

Pseudomonas aeruginosa:	ATCC	15442
	CIP	103467
	DSM	939
	NCIB	10421
Staphylococcus aureus:	ATCC	6538
	CIP	4.83
	DSM	799
	NCTC	10788
	NCIB	9518
Escherichia coli:	ATCC	10536 or 8739
	CIP	54127
	DSM	682
	NCTC	10418
	NCIMB	8879
Enterococcus hirae:	ATCC	10541
	CIP	5855
	DSM	3320
	NCIMB	8192

Annex B
(informative)

Standard test report of the measurement of the bactericidal activity of non-porous surfaces

a) Identification of the test laboratory .... XXX;

b) Reference surface used, treatment before the test and drying time

c) Identification of the test surface

product name..	Z
characteristics..	X mm x X mm x Y mm
batch number..	94-71-51
manufacturer..	Antimicrobial surfaces
date of receipt..	2018-07-11
storage conditions...	at ambient temperature and in the dark
active substance(s) and concentration (optional)	not stated

d) Test and validation method:

Number of reference and test surfaces............................ 5;

Recovery liquid..indicate whether a neutralizer is added

e) Experimental conditions:

analysis period..	15-07-2018 to 14-08-2018
test temperature	20 °C ± 1 °C
relative humidity...	45 %
Test surface preparation	*rinse with water*
Reference surface preparation	ethanol 970 ml/l *for Inox,*
contact time	*t* = 60 min ± 10 s
Drying time test surface	2 min 45 s
Drying time reference surface	2 min 30 s
incubation temperature.......................................	(36 ± 1) °C
identification of the bacterial strains used....	*Pseudomonas aeruginosa* ATCC 15442
	Staphylococcus aureus ATCC 6538
	Enterococcus hirae ATCC 10541
	Escherichia coli ATCC 10536

f) Test results: see following table

g) Conclusion:

In accordance with this document, batch 94–71–51 of surface Z (characteristics), has a 60-minute bactericidal activity, under the defined mandatory conditions, on the reference strains of *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus* and *Enterococcus hirae*.

Identified place, date and signature.

NOTE The tested product, batch number and manufacturer's name are fictive examples.

Staphylococcus aureus:

VALIDATION AND CONTROLS			**TEST SUSPENSION**		**TEST**					
	Neutralization validation **Tn**		**N**			**1**	**2**	**3**	**4**	**5**
			10^{-6}	10^{-7}						
Vc1-Vc2	10^{-1} >330->330 10^{-2} >330->330 10^{-3} 49 - 50 10^{-4} 2 - 7	10^{-1} >330->330 10^{-2} >330-325 10^{-3} 36 - 47 10^{-4} 3 - 6	317-291	32 - 41	Ref. C0	10^{0} >330->330 10^{-1} >330->330 10^{-2} 161 - 184 10^{-3} 18 - 19 10^{-4} 0 - 2	10^{0} >330->330 10^{-1} >330->330 10^{-2} 225 - 258 10^{-3} 23 - 45 10^{-4} 4 - 5	10^{0} >330->330 10^{-1} >330->330 10^{-2} 240 - 262 10^{-3} 19 - 22 10^{-4} 0 - 3	10^{0} >330->330 10^{-1} >330->330 10^{-2} >330-296 10^{-3} 22 - 28 10^{-4} 4 - 2	10^{0} >330->330 10^{-1} >330->330 10^{-2} 300 - 265 10^{-3} 31 - 32 10^{-4} 0 - 5
Log	5,70	5,53			Log	**5,24**	**5,40**	**5,39**	**5,46**	**5,46**
					Log m ± SD	**5,39 ± 0,09** difference ≤ 0,3 log				
			X = 30 955,10^{6} CFU/ml X= 8,49 log		Ref. C60 minutes	10^{0} >330->330 10^{-1} >330->330 10^{-2} 135 - 137 10^{-3} 18 - 21 10^{-4} 3 - 2	10^{0} >330->330 10^{-1} >330->330 10^{-2} 150 - 158 10^{-3} 18 - 19 10^{-4} 1 - 3	10^{0} >330->330 10^{-1} >330->330 10^{-2} 109 - 121 10^{-3} 5 - 13 10^{-4} 0 - 2	10^{0} >330->330 10^{-1} >330->330 10^{-2} 147 - 147 10^{-3} 15 - 18 10^{-4} 2 - 4	10^{0} >330->330 10^{-1} >330->330 10^{-2} 130 - 170 10^{-3} 7 - 20 10^{-4} 0 - 0
					Log	**5,15**	**5,20**	**5,06**	**5,17**	**5,18**
Mean log	5,62		N = 30 955,10^{3} CFU/µl N = 5,49 log		Log m ± SD	**5,15 ± 0,05**				
					Test T_0	F > 165 10^{0} >330->330 10^{-1} 327-300 10^{-2} 30 - 37 S: 0	F > 165 10^{0} >330->330 10^{-1} 330 - 305 10^{-2} 25 - 40 S: 0	F > 165 10^{0} >330->330 10^{-1} 260 - 286 10^{-2} 20 - 33 S: 0	F > 165 10^{0} >330->330 10^{-1} 160 - 151 10^{-2} 25 - 25 S: 0	F > 165 10^{0} >330->330 10^{-1} 264 - 277 10^{-2} 33 - 37 S: 0
					Log	**4,50**	**4,50**	**4,43**	**4,22**	**4,44**
					Log m ± SD	**4,42 ± 0,12**				
Validity	[Vn – N] ≤ 2 log difference ≤ 0,3 log		1.5.10^{8} ≤ X ≤ 5,10^{8} (X = CFU/ml) (N = CFU/µl)		Test T60 minutes	F 56 10^{0} 2 - 5 10^{-1} 0 - 0 10^{-2} 0 - 0 S: 0	F 78 10^{0} 4 - 8 10^{-1} 0 - 1 10^{-2} 0 - 0 S: 0	F 44 10^{0} 2 - 4 10^{-1} 0 - 0 10^{-2} 0 - 0 S: 0	F 62 10^{0} 3 - 6 10^{-1} 0 - 0 10^{-2} 0 - 0 S: 0	F 81 10^{0} 5 - 9 10^{-1} 0 - 2 10^{-2} 0 - 0 S: 0
					Log	**1,90**	**2,05**	**1,80**	**1,95**	**2,06**
					Log m ± SD	**1,95 ± 0,11**				
					R=C60-T60	**3,20**				

Test date: -/-/-

Annex C
(informative)

Examples of surface preparation protocols

C.1 Example 1: The surfaces are put in a beaker (minimum volume of 50 ml) containing at least 20 ml of a volume fraction of 50 ml/l Decon®[2)] for 60 minutes. Immediately rinse the disks under freshly-distilled running water for 10 s. The surface shall not dry. The disks shall only be handled with forceps. Rinse the discs with flowing water (4.2.1) for a further 10 s to ensure complete removal of the surfactant. To supply a satisfactory flow of water, a fluid dispensing pressure vessel with a hose and connectors or another suitable method can be used and regulated to supply approximately 2 000 ml per min. Disinfection is performed by placing the clean disk for 15 min in a bath containing a volume fraction of 700 ml/l isopropyl alcohol. Remove the disks and air-dry by evaporation under a laminar air flow.

C.2 Example 2: Ultrasonic cleaning can be performed for about 2 min with 50 ml of acetone. The samples are then rinsed in water and dried. Disinfection is performed by placing the clean disk for 15 min in a bath containing a volume fraction of 700 ml/l isopropyl alcohol. Remove the disks and air-dry by evaporation under a laminar air flow.

C.3 Example 3: Treat with a volume fraction of 50 ml/l Decon® for 60 min. Rinse with freshly distilled water – a volume fraction of 700 ml/l isopropyl alcohol for 15 min. Dry by evaporation under a laminar air flow.

C.4 Example 4: Rinse with freshly distilled water 700 ml/l) isopropyl alcohol for 15 minutes – Dry by evaporation under a laminar air flow.

C.5 Example 5: Treat with 30 ml/l Decon Neutracon® for 60 min. Rinse with freshly distilled water –700 ml/l isopropyl alcohol for 15 min. Dry by evaporation under a laminar air flow.

C.6 Example 6: Sonication for 5 min in ethanol 970 ml/l. Dry under a laminar air flow.

C.7 Example 7: Immerse the samples in a disinfecting solution (e.g. Aniospray® or ethanol at 70°) for 1 hour, then rinse three times in sterile distilled water, then dry under a laminar air flow for 1 hour.

C.8 Example 8: Immerse the samples in Isopropanol 998 ml/l for minimum 15 min. Remove the disks and air-dry by evaporation under a laminar air flow.

C.9 Example 9: Immerse the sample in Ethanol 700 ml/l for 15 s and wiping dry.

2) ®: Decon, Decon Neutracon and Aniospray are examples of suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.