SLOVENSKI STANDARD
SIST EN 1657:2001

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Kemična razkužila in antiseptiki - Kvantitativni suspenzijski preskus za ocenjevanje fungicidnega delovanja kemičnih razkužil in antiseptikov v veterini - Preskusna metoda in zahteve (faza 2, stopnja 1)

Chemical disinfectants and antiseptics - Quantitative suspension test for the evaluation of fungicidal activity of chemical desinfectants and antiseptics used in veterinary field - Test method and requirements (phase 2, step 1)

Chemische Desinfektionsmittel und Antiseptika - Quantitativer Suspensionsversuch zur Bestimmung der fungiziden Wirkung chemischer Desinfektionsmittel und Antiseptika für den Veterinärbereich - Prüfverfahren und Anforderungen (Phase 2, Stufe 1)

Antiseptiques et désinfectants - Essai quantitatif de suspension pour l'évaluation de l'activité fongicide des antiseptiques et des désinfectants chimiques utilisés dans le domaine vétérinaire - Méthode d'essai et prescriptions (phase 2, étape 1)

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Chemical disinfectants and antiseptics - Quantitative suspension test for the evaluation of fungicidal activity of chemical disinfectants and antiseptics used in veterinary field - Test method and requirements (phase 2, step 1)

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This European Standard was approved by CEN on 22 November 1999.

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EUROPEAN COMMITTEE FOR STANDARDIZATION
COMITÉ EUROPÉEN DE NORMALISATION
EUROPÄISCHES KOMITEE FÜR NORMUNG

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Contents

Foreword

This European Standard has been prepared by Technical Committee CEN/TC 216 "Chemical disinfectants and antiseptics", the secretariat of which is held by AFNOR.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by July 2000, and conflicting national standards shall be withdrawn at the latest by July 2000.

According to the CEN/CENELEC Internal Regulations, the national standards organizations of the following countries are bound to implement this European Standard: Austria, Belgium, Czech Republic, Denmark, Finland, France, Germany, Greece, Iceland, Ireland, Italy, Luxembourg, Netherlands, Norway, Portugal, Spain, Sweden, Switzerland and the United Kingdom.

A collaborative trial is currently being undertaken and will be used to provide a precision annex to this standard.

Annex A is normative, annexes B, C, D, E and F are informative.

Introduction

This European Standard describes a suspension test for establishing whether a chemical disinfectant or antiseptic in the veterinary field has or does not have fungicidal activity under the laboratory conditions, defined by this European Standard, which influence the action of disinfectants in practical use.

The type and level of interfering substance can be selected as well as contact times and temperatures in addition to the levels specified in order to support recommendations for use under particular conditions. Specific criteria for testing teat disinfectants are also described. The method involves neutralization of the fungicidal activity by dilution into a previously validated neutralizer. If a validated neutralizer cannot be established the method is undertaken using membrane filtration provided this has itself been previously validated for the product under test.

The conditions that shall be tested are intended to cover general purposes and to allow reference between laboratories and product types. For some applications, however, the recommendations of use of a product may differ and therefore additional test conditions need to be used.

1 Scope

This European standard specifies a test method (phase 2/step 1) and the minimum requirements for fungicidal activity of chemical disinfectant and antiseptic products that form a homogeneous physically stable preparation in standardized hard water. This European Standard is applicable to products for use in the veterinary field i.e. in the breeding, husbandry, production, transport and disposal of all animals except when in the food chain following death and entry to the processing industry.

NOTE 1 Using this European standard it is not possible to determine the fungicidal activity of an undiluted product as some dilution is always produced by adding the inoculum and interfering substance. Products can only be tested at a concentration of 80% or less.

NOTE 2 This method corresponds to a phase 2 step 1 test (see annex F).

2 Normative references

This European Standard incorporates by dated or undated reference, provisions from other publications. These normative references are cited at the appropriate places in the text and the publications are listed hereafter. For dated references, subsequent amendments to or revisions of any of these publications apply to this European Standard only when incorporated in it by amendment or revision. For undated references the latest edition of the publication referred to applies.

EN 1040:1997, *Chemical disinfectants and antiseptics - Basic bactericidal activity - Test method and requirements (phase 1).*

EN 1275:1997, *Chemical disinfectants and antiseptics - Basic fungicidal activity - Test method and requirements (phase 1).*

EN 12353, *Chemical disinfectants and antiseptics - Preservation of microbial strains used for the determination of bactericidal and fungicidal activity.*

ISO 4793, *Laboratory sintered (fritted) filters - Porosity grading - Classification and designation.*

3 Terms and definitions

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

3.1
product (for chemical disinfection and/or antisepsis)
chemical agent or formulation used as a chemical disinfectant or antiseptic [EN 1040:1997]

3.2
fungicide
product which kills fungi including their spores under defined conditions [EN 1275:1997]

NOTE The adjective derived from "fungicide" is "fungicidal".

3.3
fungicidal activity
capability of a product to produce a reduction in the number of viable vegetative yeast cells and mould spores of relevant organisms under conditions defined by this European standard

4 Requirements

The product diluted in standardized hard water when tested, in accordance with clause 5, shall demonstrate at least a 10^4 logarithmic reduction in viable counts when the test organisms are vegetative cells of *Candida albicans* and the spores of *Aspergillus niger*. The test is carried out under simulated low (3,0 g/l bovine albumin) or high

soiling conditions (10 g/l yeast extract and 10 g/l bovine albumin) according to its practical applications and under the required test conditions (10 °C (30 °C for teat disinfectants) 30 min, 2 referenced strains).

Where appropriate additional and optional contact times of 1 min ± 5 s, 5 min ± 10 s and 60 min ± 10 s and additional and optional temperatures of 4 °C, 20 °C and 40 °C are specified.

5 Test method

5.1 Principle

5.1.1 A test suspension of yeast cells and mould spores in a solution of interfering substances is added to a prepared sample of the product under test. The mixture is maintained at 10 °C ± 1 °C (or 30 °C ± 1 °C for teat disinfectants). At a contact time of 30 min ± 10 s an aliquot is taken ; the fungicidal action in this portion is immediately neutralized or suppressed by a validated method. The method of choice is dilution-neutralization. If a suitable neutralizer cannot be found, membrane filtration is used. The number of surviving yeast cells or mould spores in each sample is determined and the reduction in viable counts calculated.

Preliminary tests leading to the choice of the inactivation method should be carried out before the actual test, for each fungal strain. However, after choosing the method (dilution-neutralization or membrane filtration), it is necessary to check the inactivation of the carry over in parallel with the actual test.

5.1.2 The test is performed using vegetative cells of *Candida albicans* and spores of *Aspergillus niger*.

5.2 Materials and reagents

5.2.1 Test organisms

5.2.1.1 The fungicidal activity shall be evaluated using the following two strains :

— Candida albicans ATCC 10231 [1] ;

— Aspergillus niger ATCC 16404 [1] ;

NOTE See annex E for corresponding strain numbers in some other culture collections.

5.2.1.2 If required for specific applications additional strains may be chosen and shall be noted in the test report. Their suitability for supplying *inocula* of sufficient concentration shall be verified. If the additional strains tested are not classified at a reference centre, identification characteristics shall be given. In addition they shall be held by the testing laboratory under a reference for 5 years.

If additional strains do not grow on the medium (see **5.2.2.3**) and/or cannot be used with diluent (see **5.2.2.4**) additional media shall be used and shall be reported as well as additional cultivation conditions.

5.2.2 Culture media and reagents

5.2.2.1 General

The reagents shall be of analytical grade and/or appropriate for microbiological purposes.

NOTE To improve the reproducibility, it is recommended that commercially available dehydrated material is used for the preparation of culture media. The manufacturer's instructions relating to the preparation of these products should be rigorously followed.

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1) ATCC 10231 and ATCC 16404 are the collection numbers of strains supplied by the American Type Culture Collections. This information is given for the convenience of users of this standard and does not constitute an endorsement by CEN of the product named. Corresponding strains supplied by other culture collections may be used if they can be shown to lead to the same results.

5.2.2.2 Water

The water shall be free from substances that are toxic or inhibiting to the yeast cells and fungal spores. It shall be freshly glass distilled and not demineralised water.

Sterilize in the autoclave (see **5.3.1**).

NOTE 1 If the water is sterilized during the sterilization of the reagents this is not necessary.

NOTE 2 If distilled water of adequate quality is not available, water for injectable preparations (European Pharmacopoeia) can be used.

5.2.2.3 Malt extract agar (MEA)

Malt extract	30,0 g
Soya peptone, papaic digest of Soybean meal	3,0 g
Agar	15,0 g
Water (see **5.2.2.2**)	1 000,0 ml

Sterilize in the autoclave (see **5.3.1**). After sterilization the pH of the medium shall be equivalent to 5,6 ± 0,2 when measured at 20 °C.

5.2.2.4 Diluent

Tryptone Sodium Chloride Solution :

Tryptone, pancreatic digest of casein	1,0 g
NaCl	8,5 g
Water (see **5.2.2.2**)	1 000,0 ml

Sterilize in the autoclave (see **5.3.1**). After sterilization the pH of the medium shall be equivalent to 7,0 ± 0,2, when measured at 20 °C.

5.2.2.5 Neutralizer

The neutralizer shall be validated for the product under test in accordance with annex A. The neutralizer shall be sterile.

NOTE Information on neutralizers that have been found to be suitable for some categories of products is given in annex B.

5.2.2.6 Rinsing liquid (for membrane filtration)

The liquid shall be sterile, compatible with the filter membrane and capable of filtration through the filter membrane under the test conditions described in annex A.

NOTE Information on rinsing liquids that have been found to be suitable for some categories of products is given in annex C.

5.2.2.7 Standardized Hard water for dilution of products

Standardized hard water for dilution of products shall be prepared as follows :

— solution A : Dissolve an amount equivalent to 19,84 g anhydrous $MgCl_2$ and an amount equivalent to 46,24 g anhydrous $CaCl_2$ in water (see **5.2.2.2**) and make up to 1 000 ml. Sterilize in the autoclave (see **5.3.2.1**) ;

— solution B : Dissolve 35,02 g $NaHCO_3$ in water (see **5.2.2.2**) and dilute to 1 000 ml. Sterilize by passing through a filter with a maximum effective pore size of 0,22 µm.

Add at least 600 ml water (see **5.2.2.2**) to 6 ml of solution A in a sterile 1 000 ml volumetric flask, then add 8,0 ml solution B and make up to 1 000 ml with water (see **5.2.2.2).**

The pH of the solution shall be 7,0 ± 0,2 before use.

The water shall be freshly prepared, i.e. not used for more than one day.

NOTE When preparing the three concentrations of product test solutions (see **5.4.2**) the addition of the product in this hard water (see **5.2.2.7**) solution produces a different final water hardness in each test tube. In any case the final hardness is lower than 300 mg/kg of $CaCO_3$ in the test tube.

5.2.2.8 Interfering substances

5.2.2.8.1 General

The interfering substance shall be chosen according to the conditions of use laid down for the product.

The interfering substance shall be sterile and prepared at 10 times its final concentration in the test.

The composition shall be noted in the test report (see **5.8**).

5.2.2.8.2 Bovine albumin for low level soiling conditions

— Dissolve 3 g of bovine albumin (Cohn fraction *V* for Dubos Medium) in 90 ml of water (see **5.2.2.2**) in a 100 ml volumetric flask. Make up to the mark with water (see **5.2.2.2**) ;

— sterilize by membrane filtration.

The final concentration of the bovine albumin in the test procedure (see **5.5.2**) is 3 g/l.

5.2.2.8.3 Albumin/yeast extract mixture for high level soiling conditions

a) Dissolve 50 g yeast extract powder in 150 ml of water (see **5.2.2.2**) in a 250 ml volumetric flask and allow foam to collapse. Make up to the mark with water (see **5.2.2.2**). Transfer to a clean dry bottle and sterilize in an autoclave (see **5.3.1**). Allow to cool to 20 °C ± 5 °C.

b) Pipette 25 ml of this solution into a 50 ml volumetric flask and add 10 ml of water (see **5.2.2.2**). Dissolve 5 g of the bovine albumin (Cohn fraction V for Dubos Medium) in the solution in the flask with shaking and allow foam to collapse. Make up to the mark with water (see **5.2.2.2**) sterilize by filtration and keep in 10 ml portions in a refrigerator (at 2 °C to 8 °C) until use.

The final concentration in the test procedure (see **5.5.2**) is 10 g/l yeast extract and 10 g/l bovine albumin.

5.2.2.8.4 Milk for teat disinfectants

Skimmed milk, reconstituted at a rate of 100 g powder, guaranteed free of antibiotics or additives, per litre of water (see **5.2.2.2**), shall be prepared as follows :

— prepare a solution of 10,0 % (v/v) in water (see **5.2.2.2**) of reconstituted milk. Sterilize for 30 min at 105 $^{+3}_{0}$ °C (or 5 min at 121 $^{+3}_{0}$ °C).

The final concentration of reconstituted milk in the test procedure (see **5.5.2**) shall be 10 g/l of reconstituted milk.

5.3 Apparatus and glassware

5.3.1 General

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

a) in an autoclave (see **5.3.2.1**) by maintaining it at 121 $^{+3}_{0}$ °C for a minimum holding time of 15 min ;

b) in the dry heat sterilizer (see **5.3.2.1**) by maintaining it at 180 °C for a minimum holding time of 30 min at 170 °C for a minimum holding time of 1 h, or at 160 °C for a minimum holding time of 2 h.

5.3.2 Usual microbiological laboratory equipment [2] and, in particular, the following :

5.3.2.1 Apparatus for sterilization

a) For moist heat sterilization, an autoclave capable of being maintained at 121 $^{+3}_{0}$ °C for a minimum holding time of 15 min ;

b) for dry heat sterilization, a hot air oven capable of being maintained at 180 °C for a minimum holding time of 30 min at 170 °C for a minimum holding time of 1 h or at 160 °C for a minimum holding time of 2 h.

5.3.2.2 **Water baths**, capable of being controlled at 4 °C ± 1 °C, 10 °C ± 1 °C, 20 °C ± 1 °C, 30 °C ± 1 °C, 40 °C ± 1 °C and 45 °C ± 1 °C.

5.3.2.3 **Incubator**, capable of being controlled at either 30 °C ± 1 °C.

5.3.2.4 **pH meter,** having an accuracy of calibration of ± 0,1 pH units at 25 °C.

5.3.2.5 **Fritted filter,** porosity of 40 µm to 100 µm (see ISO 4793).

5.3.2.6 **Stopwatch**

5.3.2.7 **Vortex mixer** (electromechanical agitator, e.g. Vortex ® mixer [3])

5.3.2.8 **Membrane filtration apparatus** (if this method is used) constructed of a material compatible with the product under test, with a filter holder which shall have a usable volume of 50 ml minimum, and suitable for use with filters of diameter 47 mm to 50 mm of 0,45 µm pore size, or 0,22 µm pore size for media sterilization.

The vacuum source used shall give an even filtration flow rate. In order to obtain a uniform distribution of the micro-organisms over the membrane and in order to prevent overlong filtration, the device shall be set so as to obtain the filtration of 100 ml of rinsing liquid in 20 s to 40 s.

5.3.2.9 **Container** test tubes or flasks of suitable capacity.

5.3.2.10 **Graduated pipettes** of nominal capacities 10 ml, 1ml and 0,1 ml. Calibrated automatic pipettes may be used.

5.3.2.11 **Petri dishes** of size 90 mm to 100 mm.

5.3.2.12 **Roux bottles.**

5.3.2.13 **Glass beads** (diameter : 3 mm to 4 mm).

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2) Disposable equipment is an acceptable alternative to reusable glassware.

3) Vortex ® is an example of a suitable product available commercially. This information is given for the convenience of users of this standard and does not constitute an endorsement by CEN of this product.

5.3.2.14 **Volumetric flasks** calibrated at 20 °C.

5.3.2.15 **Centrifuge.**

5.3.1.16 **Mechanical shaker.**

5.4 Preparation of fungal suspensions and test solutions

5.4.1 Fungal suspensions

5.4.1.1 Stock cultures of test organisms

Stock cultures shall be kept in accordance with the requirements of EN 12353.

5.4.1.2 Working culture of test organisms

In order to prepare the working culture of *Candida albicans,* subculture from the stock culture (see **5.4.1.1**) by streaking onto MEA slopes (see **5.2.2.3**) and incubate (see **5.3.2.3**). After 42 h to 48 h prepare a second subculture from the first subculture in the same way and incubate for 42 h to 48 h. From this second subculture, a third subculture may be produced in the same way.

NOTE The second and/or third subcultures are the working culture(s).

If it is not possible to prepare the second subculture on a particular day, a 72 h subculture may be used for subsequent subculturing, provided that the subculture has been kept in the incubator during the 72 h period. In these circumstances, prepare a further 42 h to 48 h subculture after proceeding. Do not take a fourth subculture.

For *Aspergillus niger,* use a subculture grown on MEA (see **5.2.2.3**) in Roux bottles and incubate at 30 °C ± 1 °C for 7 to 9 days.

5.4.1.3 Fungal test suspensions

Prepare two cell suspensions, one using *Candida albicans* vegetative cells and the other using spores of *Aspergillus niger* (see **5.2.1**).

5.4.1.3.1 Preparation of yeast test suspension

Take 10 ml of diluent (see **5.2.2.4**) and place in a 100 ml flask with 10 g of glass beads (see **5.3.2.13**). Take the working cultures (see **5.4.1.2**) and transfer loopfuls of the cells into the diluent. The cells should be suspended in the diluent by immersing the loop in the diluent and rubbing it against the side of the flask to dislodge the cells. Shake the flask for 3 min using a mechanical shaker (see **5.3.2.16**). Aspirate the suspension from the glass beads and transfer to another tube. Adjust the number of cells in the suspension to 1,5 x 10^7 cfu/ml [4] to 5 x 10^7 cfu/ml using the diluent, estimating the numbers of units by any suitable means. Maintain this suspension in the water bath at 10 °C ± 1 °C and use within 2 h.

5.4.1.3.2 Preparation of *Asperigillus niger* spores test suspension

Take the working culture (see **5.4.1.2**) and suspend the cells in 10 ml of sterile 0,05 % (v/v) polysorbate 80 solution in water (see **5.2.2.2**). Using a sterile glass spatula detach the conidiospores from the culture surface. The suspension is transferred into a conical flask and gently shaken for one minute together with glass beads (see **5.3.2.13**). The suspension is filtered through a fritted filter (see **5.3.2.5**).

Microscopic examination under 400 magnification shall be carried out immediately after the preparation and just before the test, to show the absence of mycelia fragments and spore germination (less than one in ten fields of view should show either).

If germinated spores are present, the suspension shall be discarded.

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4) cfu : colony forming unit per ml.