BRITISH STANDARD

BS 6734:2004

Antimicrobial efficacy of disinfectants for veterinary and agricultural use —

Method

ICS 11.080.20; 71.100.35

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Committees responsible for this British Standard

The preparation of this British Standard was entrusted to Technical Committee, CH/216, Chemical disinfectants and antiseptics, upon which the following bodies were represented:

Association of British Healthcare Industries

British Association for Chemical Specialities

British Medical Association

British Society of Soil Science

Campden and Chorleywood Food Research

Camping and Caravanning Club Ltd

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Department for Environment, Food and Rural Affairs

Health Protection Agency

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Society for Applied Microbiology

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Trading Standards Institute

UK Cleaning Products Industry Association

Veterinary Medicines Directorate

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Contents

Foreword

This British Standard has been prepared by Technical Committee CH/216. It supersedes BS 6734:1986, which was withdrawn in April 2004 due to lack of confidence.

The concerns about BS 6734:2004 expressed by committee members have been addressed in drafting this standard.

This publication does not purport to include all the necessary provisions of a contract. Users are responsible for its correct application.

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Summary of pages

This document comprises a front cover, an inside front cover, pages i and ii, pages 1 to 9 and a back cover.

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1 Scope

This British Standard describes a method for determining the antibacterial efficacy of disinfectants intended for use in livestock farming applications.

The method described in this standard is applicable to disinfectants intended for use against tuberculosis.

2 Terms and definitions

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

2.1
antibacterial efficacy
lowest concentration (greatest dilution) of a disinfectant that will reduce the bacterial population in a challenge medium by at least a factor of 10^4

3 Principle

A challenge medium containing *Mycobacterium fortuitum* is added to a series of dilutions of a product in hard water. After a contact time of 60 min at 4 °C, a portion of each mixture is deactivated and then subcultured into five replicate tubes of recovery broth, which are incubated and examined for growth.

NOTE 1 The test organism has been placed in Category 2 by the Advisory Committee on Dangerous Pathogens.

The procedure is repeated, as necessary, until two dilutions differing in concentration by a ratio 9:10 are obtained, one of which produces growth in more than three of the five replicates and the other of which shows no growth in two or more of the five replicates.

NOTE 2 Absence of growth in two or more of the five replica cultures is equivalent to a reduction of at least 99.99 % in the initial colony count.

4 Sterilization

Wherever sterile reagents, media, materials or apparatus are referred to, or an instruction to sterilize them is given, sterility shall be that achieved by being kept at either:

a) 170 °C to 175 °C for not less than one hour in an oven (dry sterilization[1)]); or
b) 121^{+3}_{0} °C for not less than 15 min in an autoclave (wet sterilization).

Carry out manipulation of sterile material and bacterial cultures aseptically.

5 Reagents and materials

5.1 General

All reagents shall be of recognized biological or analytical grade. Water shall be free from substances that are toxic or inhibiting to the bacteria. It shall be freshly glass distilled or, if distilled water of adequate quality is not available, water for injectable preparations (EDQM) and not demineralized water.

All solutions and media other than yeast suspension shall be freshly prepared so as to be fit for purpose, i.e. support bacterial growth as appropriate and not possess inhibitory properties.

5.2 *Diluent.* Dissolve 0.305 g of anhydrous calcium chloride ($CaCl_2$) and 0.139 g of magnesium chloride hexahydrate ($MgCl_2.6H_2O$) in water and dilute to one litre.

NOTE This is standard hard water of 342 mg/kg hardness in accordance with Emulsion Stability Test WHO/M/13 in *Specifications for pesticides used in public health* [1].

1) This method is suitable for dry glassware only.

5.3 *Test organism Mycobacterium fortuitum*, NCTC 8573, NCIMB 10384[2), 3)].

5.4 *Solid culture medium.* Blood Agar Base [2), 4)]

Composition:

a) meat extract powder	10.0 g/l;
b) peptone	10.0 g/l;
c) sodium chloride	5.0 g/l;
d) agar	15.0 g/l.

Sterilize (see Clause **4**).

5.5 *Liquid culture media.*

5.5.1 *Sterile recovery broth.* Nutrient Broth No 2[2), 4)].

Composition:

a) meat extract powder	10.0 g/l;
b) peptone	10.0 g/l;
c) sodium chloride	5.0 g/l;

Sterilize (see Clause **4**).

5.5.2 *Challenge culture broth*. Add 0.05 % volume by volume polysorbate 80[2), 5)] to the liquid medium (**5.5.1**). Sterilize (see Clause **4**).

5.6 *Inactivator*. Add, aseptically, 0.5 ml of sterile horse serum to 9.5 ml portions of liquid culture medium (**5.5.1**) when cool.

5.7 *Yeast*, 250 g moist baker's yeast.

NOTE It is recommended that the yeast is obtained locally, so that it is as fresh as possible.

5.8 *Yeast suspension.* Place three suitable dishes with a reasonably large surface area (the base of a 90 mm glass Petri dish is ideal) in a drying oven at a temperature not greater than (105 ± 2) °C (**6.2**g)) and dry. Allow to cool in a desiccator. Weigh to the nearest 1 mg and record their masses. Crumble the yeast (**5.7**) by hand into a one-litre beaker and slowly add 500 ml of the diluent (**5.2**), creaming and stirring with a heavy glass rod until all the lumps are mixed in. Thoroughly stir the yeast suspension and transfer (10 ± 0.1) g of it, weighed to an accuracy of 1 mg, from the beaker to each of the dried, weighed dishes.

Place each of the dishes in a drying oven at a temperature of (105 ± 2) °C (**6.2**g)) and dry, then allow to cool in a desiccator and reweigh. Repeat these procedures until two consecutive weighings are within 1 mg. Calculate the mean dry mass of yeast as a percentage of the moist material.

NOTE Four hours in the drying oven, followed by one or two further one hour periods in the oven have been found to be satisfactory in most cases.

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2) For information on the availability of these reagents and materials, contact BSI Customer Services, British Standards House, 389 Chiswick High Road, London W4 4AL.

3) NCTC 8573 is the designation given by the National Collection of Type Cultures and NCIMB 10384 is the designation provided by the National Collection of Marine and Industrial Bacteria. This information is given for the convenience of users of this standard and does not constitute an endorsement by BSI 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.

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

5) Tween 80® 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 BSI of the product named.

During the operation to calculate the mean dry mass, place the remainder of the yeast suspension in a refrigerator at 2 °C to 8 °C.

Measure the volume of the remaining yeast suspension by transferring from the beaker to a two-litre measuring cylinder, swirling it to mix as it is poured out and making sure that no lumps remain. Record the volume (±2 ml). Calculate the volume of diluent to be added to obtain a 5 % mass by mass suspension calculated on the dry mass of the yeast. Add that amount of diluent to the cylinder using some of the diluent to wash out the beaker.

Transfer 100 ml portions into screw-capped bottles. Sterilize (see Clause **4**).

Mark the level in the bottles before autoclaving and discard those which show any excessive loss in the autoclaving process.

Allow the bottles to cool slowly and then store them in a refrigerator at 2 °C to 8 °C for at least 8 weeks and not more than 14 weeks before use. Take one bottle of the prepared yeast suspension, empty the contents into a 150 ml beaker and, by means of a pH meter, determine the pH of the suspension. Add, with stirring, sodium hydroxide solution, at a concentration of 0.2 mol/l, from a burette until a pH of 6.9 to 7.1 is obtained. Calculate the amount of sodium hydroxide solution required (±0.1 ml).

To each of the other bottles in the batch add aseptically the calculated amount of sterile 0.2 mol/l sodium hydroxide solution determined.

5.9 *Ringer's solution*, quarter-strength. Make a quarter-strength Ringer's solution in accordance with either a) or b).

a) Dissolve 9.00 g of sodium chloride, 0.42 g of potassium chloride, 0.48 g of calcium chloride hexahydrate and 0.20 g of sodium hydrogen carbonate in water and dilute to 1 000 ml. Add one volume of the solution to three volumes of water to give a quarter-strength solution.

b) Dissolve one quarter-strength Ringer's solution tablet in the appropriate volume of water, to obtain a quarter-strength solution.

Then, in common for both solutions, sterilize (see Clause **4**). Prior to use, dispense (9 ± 0.1) ml volumes into suitable sterile containers (e.g. test tubes with closures, Universal containers).

6 Apparatus

6.1 Cleanliness

The standard of cleanliness normally associated with bacteriological procedures is required but, when disinfectants based on quaternary ammonium compounds have been used, special care in the cleaning of glassware is necessary. The following cleaning procedure is satisfactory and shall be used in cases of dispute, but otherwise, it is permissible for any method that can be shown to give equivalent results and is known to be satisfactory to be used.

Wash glassware in cold, 62 g/l nitric acid solution and allow to stand overnight in the acid. Rinse successively with 4 g/l sodium hydroxide solution, tap water, 6 g/l nitric acid solution and tap water until the acid is removed. Finally, rinse with distilled water.

6.2 *Ordinary microbiological laboratory apparatus*, is required especially:

a) *pipettes*, 1.0 ml graduated at 0.01 ml intervals or *automatic pipettes with sterile barrier tips*, capable of dispensing 0.1 ml and 1.0 ml accurately;

b) *pipettes*, 10 ml graduated at 0.1 ml intervals or an *automatic pipette with a sterile tip*, capable of dispensing 2.5 ml and 10 ml accurately;

c) *universal container culture bottles*, 25 ml to 35 ml;

d) *test tubes*, 150 mm × 19 mm diameter with suitable plastic caps;

e) *water bath*, capable of being controlled at (4 ± 0.5) °C and between 45 °C and 50 °C;

f) *incubator*, capable of being controlled at (37 ± 1) °C;

g) *drying oven*, capable of being controlled at (105 ± 2) °C;

h) *electromechanical agitator*, e.g. Vortex® mixer[6)];

i) *balance*, capable of reading to 1 mg;

j) *timer*, capable of reading to 1 s;

k) *graduated stoppered measuring cylinders*;

l) *refrigerator*, capable of maintaining a temperature of 2 °C to 8 °C;

m) *pH meter*, capable of reading to 0.01 pH units;

n) *bacteriological loop*.

Measuring equipment shall be calibrated as appropriate.

NOTE Universal container culture bottles c) may be used instead of test tubes d).

7 Preparation of test culture

7.1 Initial culture

The test organism (**5.3**), distributed in freeze-dried form, shall be opened in accordance with the supplier's instructions.

Using a sterilized pipette, add approximately 0.5 ml of the sterilized challenge culture broth (**5.5.2**) to the contents of the tube and incubate for seven days in the incubator (**6.2**f)) controlled at (37 ± 1) °C.

From this initial culture, prepare the stock culture (**7.2**) and, from that, the broth cultures (**7.3**).

7.2 Stock culture

Spread a loopful of the initial culture (**7.1**) over the surface of a slope of the sterilized solid culture medium (**5.4**) in a universal bottle (**6.2**c)). Incubate for seven days in the incubator (**6.2**f)) controlled at (37 ± 1) °C, and store in a refrigerator between 2 °C and 8 °C until required. Use stock cultures so stored within one month, although subcultures can be taken before the expiry of that date and used within another month. However, do not take more than six serial subcultures before resorting to a new freeze-dried culture (**5.3**).

6) 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 BSI of the product named.

7.3 Broth culture

Inoculate a 10 ml volume of the challenge culture broth (**5.5.2**) in a test tube or universal container (**6.2**c) or d)) from the stock culture (**7.2**) and incubate in the incubator (**6.2**f)) at (37 ± 1) °C. Progressively subculture into fresh challenge culture broth every seven days, incubating the inoculum at (37 ± 1) °C for 7 days ± 4 h.

Use subcultures 1 to 4 for the preparation of the challenge medium (Clause **8**). After four broth-to-broth subcultures, restart the process using a fresh stock culture (**7.2**).

7.4 Colony count

Immediately before preparing the challenge medium (Clause **8**) make serial decimal dilutions of the broth culture (**7.3**) in the quarter-strength Ringer's solution (**5.9**), mixing before taking samples. Prepare duplicate pour plates from 1 ml portions of the sixth, seventh and eighth dilutions and 15 ml portions of the sterilized solid culture medium (**5.4**) after the latter has been melted and equilibrated at 45 to 50 °C. Allow to set. Invert the plates and incubate in the incubator (**6.2**f)), controlled at (37 ± 1) °C, in the inverted position for 7 days and then count the colonies. If the colonies appear atypical or if the broth culture (**7.3**) appears atypical or contains less than 10^8 colony-forming units per millilitre, consider the test void and repeat the test from the beginning.

8 Preparation of challenge medium

Take (4 ± 0.01) ml of broth culture (**7.3**) and add to (96 ± 0.05) ml of the yeast suspension (**5.8**). Use this mixture within two hours. Equilibrate at (4 ± 0.5) °C in the water bath (**6.2**e)).

NOTE This equilibration can usually be obtained by cooling in the water bath for at least 20 min.

9 Preparation of dilutions

9.1 General

Prepare the test dilutions using the following methods. Prepare initial dilutions of both liquid and solid products gravimetrically to provide accurate and comparable results for all products, irrespective of whether they are solid or liquid and how viscous the liquids are.

If the potency of the product is known accurately, the test may be adapted so that the product is tested at fewer than five concentrations.

9.2 Liquid products

The recommended use dilution of the disinfectant is D_l, where 1 ml of the liquid product is effective when added to D_l ml of water.

Where D_l is known, ascertain the density of the product. Measure the tare weight of a 100 ml volumetric flask. Fill to the mark with product, record the weight and hence calculate the density of the product in g/ml.

Prepare a dilution of the disinfectant in the diluent (**5.2**) gravimetrically, on a mass per volume basis, equivalent to a concentration 123 % volume by volume of D_l. To do this, measure the tare weight of a suitable vessel and then add a mass of the disinfectant calculated as:

$$A_l = \frac{1.23 \times \rho \times 150}{D_l}$$

where

A_l is the mass of liquid disinfectant required;

ρ is the density of the disinfectant;

D_l is the recommended use dilution of the liquid disinfectant.

Then add 150 ml of diluent (**5.2**).

9.3 Solid products

The recommended use dilution of the disinfectant is D_s, where 1 g of the solid product is effective when added to D_s ml of water.

Where D_s is known, prepare a dilution of the disinfectant in the diluent (**5.2**) gravimetrically to a concentration 123 % mass by volume of D_s. To do this, measure the tare weight of a suitable vessel and then add a mass of the disinfectant calculated as:

$$A_s = \frac{1.23 \times 150}{D_s}$$

where

A_s is the mass of solid disinfectant required;

D_s is the recommended use dilution of the solid disinfectant.

Then add 150 ml of diluent (**5.2**).

9.4 No recommended use dilution

Where the product has a completely unknown potency, carry out some preliminary tests on a series of widely ranged dilutions and repeat until the aim of the test (see Clause **3**) is achieved.

9.5 Dilution of products

Mix thoroughly the primary solution (**9.2**, **9.3** or **9.4**) and then measure 100 ml into a stoppered graduated cylinder. Use this first dilution in the test procedure (Clause **10**) within two hours of preparation. Using a 10 ml graduated-capacity pipette or automatic pipette (**6.2**b)), transfer 10 ml to a clean universal bottle and top up the liquid in the measuring cylinder with 10 ml of diluent (**5.2**) and mix thoroughly. Again, using an air displacement pipette, remove a further 10 ml volume to another universal bottle and repeat this process until five universal bottles, each containing 10 ml of the disinfectant, diluted to 123 %, 110 %, 100 %, 90 % and 80 % of the recommended use dilution are available. Using a graduated-capacity pipette or automatic pipette (**6.2**b)), transfer a 2.5 ml portion of each of these serially diluted solutions into a test tube or universal bottle (**6.2**c) or d)). Equilibrate these at (4 ± 0.5) °C in the water bath (**6.2**e)). If a satisfactory result is not obtained using these dilutions, proceed to a range of higher or lower concentrations.

10 Test procedure

10.1 At zero time

Using a graduated capacity or automatic pipette, (**6.2**b)), add 2.5 ml of the challenge medium (Clause **8**) equilibrated to (4 ± 0.5) °C to the tube containing 2.5 ml of the disinfectant solution at 123 % of the recommended use dilution. Mix the contents of the tube thoroughly and transfer to a fresh sterile tube, appropriately numbered and cooled to (4 ± 0.5) °C. After transfer replace the tube in the water bath.

Repeat this operation four times, using the other disinfectant solutions, at 1 min intervals. Shake the tubes at 10 min intervals.

10.2 After 60 min

Remove the first tube from the water bath, mix thoroughly, transfer 0.1 ml to 10 ml of the inactivator (**5.6**) using a 1.0 ml graduated-capacity or automatic pipette (**6.2**a)) and mix thoroughly. Repeat at one-minute intervals thereafter until all tubes have been sampled. Leave these inactivated preparations to stand for at least five minutes.

Using a pipette (**6.2**b)) transfer 1 ml portions from the first inactivated preparation to each of five tubes containing 10 ml of the sterile recovery broth (**5.5.1**). Continue, in sequence, transferring five 1 ml portions of the inactivated preparations from each tube to separate 10 ml recovery broths, using a fresh pipette for each inactivated preparation. Complete all these transfers of the inactivated preparations to the recovery broth within 20 min of the sampling and preparation of the inactivated mixture.

10.3 Incubate the tubes at (37 ± 1) °C for 7 days ± 4 h.

10.4 Examine the tubes and record those showing evidence of growth, such as turbidity or the presence of "breadcrumbs". In case of doubt, or if growth is atypical, confirm by extending the incubation period for a further seven days, or by streaking out a sample containing the possible growth onto 15 ml portions of the sterilized solid culture medium (**5.4**). Invert the plates and incubate in the incubator (**6.2**f)), controlled at (37 ± 1) °C, in the inverted position for up to seven days.

10.5 Prepare a negative control sample by adding 0.1 ml of the yeast suspension (**5.8**) and 1.0 ml of the inactivator (**5.6**) to a tube containing 10 ml of the sterile recovery broth (**5.5.1**). Incubate the tube at (37 ± 1) °C for 7 days ± 4 h. If this broth shows increased turbidity, the test is void and shall be repeated. In case of doubt, confirm by subculture or further incubation.

10.6 Prepare a positive control sample by adding 0.1 ml of the broth culture (**7.3**) and 1.0 ml of the inactivator (**5.6**) to a tube containing 10 ml of the sterile recovery broth (**5.5.1**). Incubate the tube at (37 ± 1) °C for 7 days ± 4 h. If this broth does not show increased turbidity, the test is void; repeat the test in this case.

11 Test result

For a liquid product, record the antibacterial efficacy in terms of the greatest dilution of the product in volume by volume terms that produces absence of growth in two or more of the resulting five replicate cultures. For a solid product, record the results in mass by volume terms.

12 Test report

Include the following items in the test report:

a) a reference to this British Standard, i.e. BS 6734:2004;

b) an identification of the laboratory sample;

c) the batch number or other specific identification of the product;

d) the result determined as in Clause **11**;

e) the date of the test.

Bibliography

[1] *Specifications for pesticides used in public health*. 4th ed. Geneva: World Health Organization, 1973, **304**.

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