

NATIONAL STANDARD METHOD

*UNDER CONSULTATION*  
**SUSCEPTIBILITY  
TESTING**

**BSOP 45**

Issued by Standards Unit, Department for Evaluations, Standards and Training  
**Centre for Infections**



*Association of Medical Microbiologists*  
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**SUSCEPTIBILITY TESTING**

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# STATUS OF NATIONAL STANDARD METHODS

National Standard Methods, which include standard operating procedures (SOPs), algorithms and guidance notes, promote high quality practices and help to assure the comparability of diagnostic information obtained in different laboratories. This in turn facilitates standardisation of surveillance underpinned by research, development and audit and promotes public health and patient confidence in their healthcare services. The methods are well referenced and represent a good minimum standard for clinical and public health microbiology. However, in using National Standard Methods, laboratories should take account of local requirements and may need to undertake additional investigations. The methods also provide a reference point for method development.

National Standard Methods are developed, reviewed and updated through an open and wide consultation process where the views of all participants are considered and the resulting documents reflect the majority agreement of contributors.

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The reader is informed that all taxonomy in this document was correct at time of issue.

*Please note the references are now formatted using Reference Manager software. If you alter or delete text without Reference Manager installed on your computer, the references will not be updated automatically.*

## **Suggested citation for this document:**

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# AMENDMENT PROCEDURE

Controlled document reference	BSOP 45
Controlled document title	Susceptibility testing

Each National Standard Method has an individual record of amendments. The current amendments are listed on this page. The amendment history is available from [standards@hpa.org.uk](mailto:standards@hpa.org.uk).

On issue of revised or new pages each controlled document should be updated by the copyholder in the laboratory.

Amendment Number/ Date	Issue no. Discarded	Insert Issue no.	Page	Section(s) involved	Amendment
3/ 30.10.06	1.2	2			

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# SUSCEPTIBILITY TESTING

**Type(s) of specimen(s):** Isolates from all specimens  
Urine specimens for direct testing  
CSF specimens for direct testing  
Positive blood cultures

## SCOPE OF DOCUMENT

This document describes routine antimicrobial susceptibility testing methods recommended for use with commonly isolated bacteria. Methods for *Mycobacterium* species, *Brucella* species and filamentous fungi are not included in this SOP. It is recommended that filamentous fungi, *Brucella* species and *Mycobacterium* species are referred to a reference laboratory for susceptibility testing.

## INTRODUCTION<sup>2</sup>

### The purpose of susceptibility testing

Susceptibility testing is required to assist the clinician in the choice of agents for therapeutic or prophylactic use. In practice, agents are often used empirically and the susceptibility test may help to explain failure to respond to therapy and indicate appropriate alternatives. Recent accumulated data on the antimicrobial susceptibility of local isolates provides information needed for selection of agents for empirical use. In addition, testing is of value for surveillance purposes, although there are limitations of surveillance based on routine test results in relation to denominators, reproducibility and the availability of quantitative results. Some bacteria currently have predictable susceptibility to some agents eg all *Streptococcus pyogenes* are susceptible to penicillin. However, susceptibility may change and routine testing is appropriate to provide early warning of developing resistance. Treatment failure may indicate a resistant strain, but it may also occur with susceptible strains because of other clinical problems, some unrelated to the infection. Treatment failure alone should not be relied upon to detect resistant strains. Susceptibility tests can also be useful in epidemiological studies, where the antibiogram may be a phenotypic characteristic which assists in typing organisms.

Identification tests are usually performed at the same time as susceptibility testing when clinically significant organisms are selected from primary isolation plates. Preliminary identification is usually the basis of selection of agents to test, and identification may alert the worker to possible difficulties in testing or interpretation. Identification and susceptibility results should be reviewed together as interpretation may be affected by the identification of the organism.

Isolated colonies should be selected from primary isolation plates for susceptibility testing. With the exception of urine, blood culture and CSF specimens, direct susceptibility testing from a specimen is not recommended (see section 2.1.4). If susceptibility testing is performed directly from specimens, pure semi-confluent growth may not be obtained. Results from susceptibility testing of mixed cultures or tests with incorrect inocula may be misleading and should be repeated with a pure culture.

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## Categories of susceptibility

The terms susceptible (sensitive), intermediate and resistant are used to indicate whether a particular antimicrobial agent is likely to be therapeutically effective against a particular organism.

**Susceptible:** This implies that an infection due to the strain may be appropriately treated with the dosage of antimicrobial agent recommended for that type of infection and infecting species, unless otherwise contraindicated. This category may include strains which are clinically susceptible but have a mechanism conferring low level resistance.

**Intermediate:** This category includes isolates with antimicrobial MICs that approach usually attainable blood and tissue concentrations and for which response to chemotherapy may be variable. Organisms with intermediate susceptibility may be eliminated from body sites where the drugs are concentrated (eg quinolones and  $\beta$ -lactams in urine) or when an increased dosage of a drug can be used (eg  $\beta$ -lactams). The intermediate category may also be taken to represent a buffer zone which should prevent small, uncontrolled, technical variations from causing major discrepancies in interpretation, especially for drugs with narrow pharmacotoxicity margins. In practice, the intermediate category is often not reported, except with a few antibiotic combinations, and the intermediate and resistant categories are combined as resistant to avoid uncertainty.

**Resistant:** Strains in this category are not inhibited by the usually achievable concentrations of the agent at the site of infection and/or have specific resistance mechanisms (eg  $\beta$ -lactamases). Therapy is highly likely to fail.

## Susceptibility breakpoints

Susceptibility breakpoints are the concentrations of antimicrobial agents or, in the case of disc diffusion tests, zone diameters which distinguish the different categories of susceptibility (susceptible, intermediate and resistant). The minimum inhibitory concentration (MIC) breakpoints are based on a knowledge of pharmacokinetics/pharmacodynamics, MIC distributions, resistance mechanisms and clinical experience of the use of a particular agent against that organism in particular infections. Zone diameter breakpoints in standardised methods are based on the correlation of zone sizes with MICs by statistical methods, the distribution of susceptibility for different species, and clinical experience.

Different breakpoints may be used by different national committees, and those of the BSAC are recommended (<http://www.BSAC.org.uk>). The BSAC are currently part of the European Committee on Antimicrobial Susceptibility Testing (EUCAST), which is involved in setting breakpoints for new agents as part of the licensing process of the European Medicines Evaluation Agency (EMA) and is currently in the process of reviewing existing breakpoints so that they are harmonised in Europe (<http://www.EUCAST.org>).

## Susceptibility testing methods

A variety of methods are available for antimicrobial susceptibility testing. Choice of method for routine use is based on the type of organism, required accuracy, technical simplicity, applicability to working practices in the individual laboratory and cost.

**Disc diffusion:** The disc diffusion method is the most widely used technique in the UK and is suitable for testing rapidly growing and certain fastidious bacterial pathogens. The surface of an agar plate is inoculated with a standardised inoculum of the test organism. Paper discs containing an appropriate amount of the test agent are placed on the plate. The agent diffuses into the medium and produces a concentration gradient with a high concentration close to the disc and a reducing concentration moving away from the disc. On incubation, a zone of inhibition of growth is formed and zone diameters are interpreted as categories of susceptibility.

In the Stokes method<sup>3</sup>, which was formerly widely used in the UK, a control strain is inoculated adjacent to the test organism on the same plate. Variation in the test is assumed to affect the control and test similarly and is cancelled out by interpretation of susceptibility by comparison of test and control zones. There are limitations to the effectiveness of control by this method<sup>4</sup>. In addition, the Stokes method has not been systematically updated for some of the new, highly active antimicrobial agents or particular resistance mechanisms and the method is not recommended. Many countries

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have nationally recognised disc diffusion methods in which variation is controlled by rigid standardisation of the test. With such methods new resistances can be accommodated by modification of interpretive criteria if necessary and new agents have interpretive criteria established for the standardised technique. Zone diameter breakpoints are valid only for the standardised method with which they were developed, and *ad hoc* modifications are likely to lead to erroneous results. The British Society for Antimicrobial Chemotherapy (BSAC) has published a standardised disc diffusion method<sup>5,6</sup> which has now been adopted by most laboratories in the UK.

**Dilution methods:** In dilution methods, standard inocula of the test organisms are exposed to a range of concentrations (conventionally a two-fold dilution series based on 1mg/L) of the antimicrobial agent. After incubation, the presence or absence of growth is assessed and the MIC of the agent is defined as the lowest concentration inhibiting visible growth. The antimicrobial agent may be incorporated in agar (agar dilution) or broth (broth dilution). There is close agreement among national authorities with regard to details of the agar dilution method except for the medium used, and full details have been published<sup>7,8</sup>. The broth macrodilution method is not widely used but versions based on microwell plates (broth microdilution<sup>8</sup>) have been widely used in some countries because they are amenable to automation and are the basis of some commercial systems. A European (CEN) and International (ISO) reference method for determination of MICs by broth microdilution is likely to be agreed early in 2006.

**Breakpoint methods:** Breakpoint methods are based on standard dilution methods but test only a very limited number of concentrations, usually the concentration or concentrations equivalent to the breakpoint concentration(s) distinguishing different categories of susceptibility. Breakpoint methods are particularly suited to laboratories processing large numbers of samples. In the UK, agar incorporation breakpoint methods are used for susceptibility tests in a few laboratories, although other methods are also used for some tests. Breakpoint methods have advantages in that they are suitable for mechanisation of inoculation and reading, can be combined with identification tests, are economical for testing large numbers of isolates, and usually have clear endpoints<sup>9</sup>. The main limitations are lack of standardisation among laboratories, the all-or-none result which masks finer degrees of susceptibility, and the difficulty in effectively controlling tests. This method is described in this National Standard Method as an alternative to the disc diffusion method.

A variation of the agar-based breakpoint method may also be used for screening for MRSA (see BSOP 29 - Investigation of specimens for screening for MRSA) or vancomycin resistant enterococci<sup>10</sup>.

**Etest:** The Etest is an MIC method<sup>11,12</sup> (an extensive reference list is available from the supplier). It consists of a 60 mm x 5 mm plastic strip with an exponential antimicrobial gradient dried on one side and an MIC scale printed on the other. The gradient of agent covers a concentration range of 0.002 to 32 mg/L, 0.016 to 256 mg/L or 0.064 to 1024 mg/L, depending upon the agent. This range corresponds to 15 two-fold dilutions in a conventional MIC method.

Tests are set up in a similar way to disc diffusion tests except that the disc is replaced with the Etest strip and inocula are heavier. After overnight incubation the MIC is read at the point of intersection of the elliptical zone with the strip. For most tests the Etest gives similar results to the agar dilution method although, as with other methods, a standardised technique should be used and care must be taken in reading results. The method is useful in routine laboratories for confirmation of unusual resistances, checking equivocal results, for testing slower-growing organisms (which do not give clearly visible growth after overnight incubation) and for organisms where a quantitative result is desirable, such as in cases of endocarditis (see section 3).

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**Automated methods<sup>13,14</sup>:** Automated methods for the determination of antibiotic susceptibility are available and have the advantages of standardisation, a wide range of agents in each test, more rapid results for many tests and some combine identification with susceptibility tests. They have the disadvantages of relatively high cost and lack of versatility.

**Detection of mechanisms of resistance:** For practical reasons, direct detection of resistance mechanisms is generally limited to  $\beta$ -lactamase testing of some fastidious organisms. The result of a rapid  $\beta$ -lactamase test may be clinically useful before the results of overnight susceptibility testing become available (see section 2.7). If rapid identification or confirmation of methicillin resistance in *S. aureus* is required, colonies may be tested for the presence of PBP2a by a latex agglutination method<sup>15</sup>.

**Molecular methods<sup>16,17</sup>:** Nucleic acid probes and PCR methods have been described for detection of many resistance genes, but in most cases the methods are at present not suitable for routine testing. Commercial methods are available for the detection of methicillin resistance in *S. aureus* and there is particular interest in using such methods for direct detection of MRSA in screening specimens rather than routine susceptibility testing on isolated organisms<sup>18,19</sup>. Further commercial developments may alter this situation in the future but the limitations of molecular methods should be recognised, particularly with regard to direct detection of resistance genes in specimens rather than isolated organisms. It is often necessary to know the identity of the infecting organism as well as its susceptibility, commensal flora may carry the same resistance genes as infecting organisms, more than one gene may confer resistance to the same agent, only targeted genes will be detected, spontaneous mutations may not be detected, and detected genes may not be expressed.

**Recommended methods:** The methods recommended in this National Standard Method are disc diffusion by the BSAC standardised method, a breakpoint method in agar, and the Etest for MIC determination. In addition,  $\beta$ -lactamase testing is recommended for *Haemophilus influenzae*, *Moraxella catarrhalis* and *Neisseria gonorrhoeae*.

## Selection of antimicrobial agents to test

All agents should be referred to by their generic names rather than their commercial names. Many antimicrobial agents are available so there is inevitably a need to be selective in testing and reporting. Appropriate antimicrobial agents are selected for testing by each clinical laboratory in consultation with clinical staff, the pharmacy and infection control teams. Selection of appropriate agents for testing is based on clinical indications, efficacy, pharmacological factors, local prevalence of resistant organisms and cost. These choices are often not clear-cut, as exemplified by use of different agents in similar clinical situations in different hospitals.

Only one agent needs to be tested where several agents in a class have similar activity, but it should be noted that the results are not as precise as when agents are tested individually. In practice, the number of agents used from a particular class is likely to be limited in a single hospital and it is preferable to test the agent used. The exceptions are where the agent tested gives a more reliable detection of resistance than the agent used clinically. For example, methicillin, oxacillin or cefoxitin are used to test staphylococci for resistance to penicillinase-resistant penicillins and cephalosporins; oxacillin is used to detect reduced susceptibility to penicillin in pneumococci; cefpodoxime, or ceftazidime plus cefotaxime are used as screening tests for extended-spectrum  $\beta$ -lactamases (ESBLs) in Enterobacteriaceae.

## Quality assurance<sup>20</sup>

In all methods, daily control of performance is achieved by use of appropriate control strains (see following sections on individual methods). With all methods, new batches of reagents (media, antimicrobial discs etc) should be tested with appropriate control strains before they are put to routine use.

Monitoring of routine results provides valuable additional control. Impossible results (eg penicillin susceptible, methicillin resistant *Staphylococcus* species), previously unknown resistances (eg penicillin resistant *S. pyogenes*) or unusual resistances (eg imipenem resistant Enterobacteriaceae) may indicate problems in susceptibility testing<sup>21</sup>. Such details may be incorporated as rules in computer systems and extensive rule bases may be part of expert systems in devices such as zone

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readers and automated broth-based susceptibility systems. Interpretative reading extends such rules by inferring a resistance mechanism from the results of tests and then reporting results based on the known effect of a resistance mechanism on clinical resistance to a range of agents<sup>21</sup>. However, the implementation of interpretative reading is restricted by the limited range of agents commonly tested in laboratories in the UK.

## TECHNICAL INFORMATION/LIMITATIONS

Methods for *Mycobacterium* species, *Brucella* species and filamentous fungi are not included in this National Standard Method.

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# 1 SAFETY CONSIDERATIONS<sup>22-27</sup>

Containment Level 2 unless infection with a Hazard Group 3 organism is suspected, in which case a microbiological safety cabinet in a Containment Level 3 room is required.

Laboratory procedures that give rise to infectious aerosols must be conducted in a microbiological safety cabinet.

Refer to current guidance on the safe handling of all organisms documented in this document.

The above guidance should be supplemented with local COSHH and risk assessments.

Compliance with postal and transport regulations is essential.

## 2 SPECIMEN PROCESSING

### 2.1 DISC DIFFUSION METHOD (BSAC)<sup>5,6</sup>

#### 2.1.1 MEDIUM

Prepare Iso-Sensitest agar (ISA) (Oxoid CM471 or equivalent medium shown to have the same performance as ISA) according to the manufacturer's instructions. Supplement media for fastidious organisms with 5% whole horse blood or 5% whole horse blood containing 20 mg/L  $\beta$ -nicotinamide adenine dinucleotide (NAD) (eg Mast Group, Merseyside) as follows:

Species	Medium
Enterobacteriaceae	ISA
<i>Pseudomonas</i> species	ISA
<i>Stenotrophomonas maltophilia</i>	ISA
Staphylococci (agents other than methicillin/oxacillin)	ISA
<i>Staphylococcus aureus</i> using cefoxitin to detect methicillin/oxacillin/cefoxitin resistance	ISA
<i>Staphylococcus aureus</i> using methicillin or oxacillin to detect methicillin/oxacillin/cefoxitin resistance	Colombia agar (Oxoid CM331 or equivalent) with 2% NaCl
Enterococci	ISA
<i>Streptococcus pneumoniae</i>	ISA + 5% defibrinated horse blood
$\alpha$ -haemolytic streptococci	ISA + 5% defibrinated horse blood + 20 mg/L NAD
$\beta$ -haemolytic streptococci	ISA + 5% defibrinated horse blood
<i>M. catarrhalis</i>	ISA + 5% defibrinated horse blood
<i>Neisseria meningitidis</i>	ISA + 5% whole horse blood
<i>Haemophilus</i> species	ISA + 5% defibrinated horse blood + 20 mg/L NAD
<i>N. gonorrhoeae</i>	ISA + 5% defibrinated horse blood
<i>N. meningitidis</i>	ISA + 5% defibrinated horse blood
<i>P. multocida</i>	ISA + 5% defibrinated horse blood

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	+ 20 mg/L NAD
<i>B. fragilis</i> , <i>B. thetaiotaomicron</i> , <i>C. perfringens</i>	ISA + 5% defibrinated horse blood + 20 mg/L NAD
<i>Campylobacter</i> species.	ISA + 5% defibrinated horse blood
Coryneform organisms	ISA + 5% defibrinated horse blood + 20 mg/L NAD
Other organisms	See sections 2.4-2.6

In order to reduce the number of different media used, ISA + 5% whole horse blood + 20 mg/L NAD may be used for all fastidious organisms listed above. For other organisms see recommendations in sections 2.4-2.6. If fastidious organisms do not grow on recommended media it may be necessary to send them to a reference laboratory.

### 2.1.2 PREPARATION OF PLATES

Pour sufficient molten agar in 90 mm sterile Petri dishes to give a depth of 4 mm ± 0.5 mm and allow to set on a level surface. Thin or uneven plates will result in variation in zone sizes. Dry the plates until the surface of the agar is free of visible surface moisture but do not overdry. Store the plates under appropriate conditions. Plate drying, storage conditions and shelf life should be determined by media production departments and controlled as part of the media quality assurance programme.

### 2.1.3 PREPARATION OF INOCULUM

Inoculum is probably the most poorly controlled variable in susceptibility testing and can have a marked effect on zone sizes. A heavy inoculum may lead to false resistance and vice versa with a light inoculum.

For most organisms the inoculum used should give semi-confluent growth of colonies after overnight incubation<sup>5</sup>. An incorrect inoculum (confluent growth or separated colonies) can be readily seen and tests with incorrect inocula should be repeated. See sections 2.4-2.6 for specific requirements relating to particular groups of organisms.

The means by which the correct inoculum is achieved is not critical as long as semi-confluent growth is obtained. Choice of method is likely to be governed by practical considerations but success will be greater if some form of standardisation is used, eg comparison of the density of suspensions of organisms with that of a 0.5 McFarland turbidity standard or latex equivalent, or use of a simple photometric method. These methods have been shown to work well and are described below.

#### Standardisation with McFarland standard

The 0.5 McFarland standard may be purchased, or prepared by adding 0.5 mL of 0.048 M BaCl<sub>2</sub> (1.17%w/v BaCl<sub>2</sub>.2H<sub>2</sub>O) to 99.5 mL of 0.18 M H<sub>2</sub>SO<sub>4</sub> (1% w/v) with constant stirring. Using matched cuvettes with a 1 cm light path and water as a blank standard, measure the absorbance in a spectrophotometer at a wavelength of 625 nm. The acceptable range for the standard is 0.08 - 0.13. Distribute the standard among screw-capped tubes of the same size and volume as those used in growing the broth culture or suspending the organism. Seal the tubes tightly to prevent evaporation and store them in the dark at ambient temperature for up to six months. Vigorously mix the standard before use.

Select colonies (when possible a minimum of four) from an overnight plated culture on non-selective medium. Inoculate the colonies to sterile distilled water or Iso-Sensitest broth (or similar medium) to produce a suspension which matches the turbidity of the 0.5 McFarland standard. This method may be used with any organisms but is the method of choice for fastidious organisms. An alternative for non-fastidious organisms is to transfer colonial growth to Iso-Sensitest broth (or similar medium) and grow at 35°C-37°C (growth will be more rapid if cultures are shaken) until the turbidity is equivalent to or exceeds that of the 0.5 McFarland standard. Compare the test and standard against a white background with a contrasting black

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line and, if necessary, adjust the density of the suspension by adding distilled water. This suspension should contain approximately  $10^8$  cfu/mL and be used within 15 minutes of preparation.

Dilute the prepared suspension in sterile distilled water before inoculation as follows:

1:100	1:10	No dilution
$\beta$ -Haemolytic streptococci	<i>Staphylococcus</i> species	<i>N. gonorrhoeae</i>
Enterococci	<i>S. pneumoniae</i>	<i>Campylobacter</i> species
Enterobacteriaceae	<i>N. meningitidis</i>	
<i>Pseudomonas</i> species	<i>M. catarrhalis</i>	
<i>S. maltophilia</i>	$\alpha$ -haemolytic streptococci	
<i>Acinetobacter</i> species	<i>C. perfringens</i>	
<i>Haemophilus</i> species	Coryneform organisms	
<i>P. multocida</i>		
<i>B. fragilis</i>		
<i>B. thetaiotaomicron</i>		

### Standardisation with spectrophotometer

Suspend colonies (select at least four when possible) in 3 mL distilled water or broth in a 4 x 1/2" glass tube to give an even suspension with just visible turbidity. Do not leave the organisms standing in water. Zero the spectrophotometer with a water or sterile broth (same as suspending medium) blank at a wavelength of 500 nm. Measure the absorbance of the bacterial suspension. From the table select the volume to transfer (with appropriate fixed volume micropipette) to 5 mL sterile distilled water. Different sized tubes or cuvettes may be used and spectrophotometers may differ slightly, so it may be necessary to adjust the dilutions slightly to achieve semi-confluent growth with any individual set of laboratory conditions. A nephelometer may be used but it will be necessary to calibrate the instrument for different groups of organisms.

Organisms	Absorbance reading at 500 nm	Volume ( $\mu$ L) to transfer to 5ml sterile distilled water
Enterobacteriaceae	0.01-0.05	250
<i>Enterococcus</i> species	>0.05-0.1	125
<i>Pseudomonas</i> species	>0.1-0.3	40
<i>Staphylococcus</i> species	>0.3-0.6	20
	>0.6-1	10
<i>Haemophilus</i> species	0.01-0.05	500
streptococci	>0.05-0.1	250
Miscellaneous fastidious organisms	>0.1-0.3	125
	>0.3-0.6	80
	>0.6-1	40

Use the adjusted suspension within 15 minutes to inoculate plates.

#### 2.1.4 INOCULATION

Dip a dry sterile cotton swab in the suspension and remove excess fluid by turning the swab against the inside of the tube. Spread the inoculum evenly over the surface of the agar plate by swabbing in three different directions. Alternative means of inoculation may be used as long as they give even growth without streaks on the plate. Before applying discs allow the plate to dry until there is no visible surface moisture.

For direct susceptibility testing of urine specimens transfer sufficient volume to the centre of the plate to give semi-confluent growth and use a sterile cotton swab to spread the inoculum

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as described above. Alternative procedures may be used as long as semi-confluent growth is achieved<sup>5</sup>.

For direct sensitivity testing of blood cultures and CSF specimens add sufficient volume (of the positive blood culture or the suspended CSF deposit) to the centre of the plate to give semi-confluent growth and spread with a sterile cotton swab as described above. Alternative procedures may be used as long as semi-confluent growth is achieved<sup>5</sup>.

### 2.1.5 DISC CONTENTS

Disc contents are as specified for the BSAC method<sup>5,6</sup> and are listed in Appendices 1 and 2. See the BSAC website<sup>6</sup> for latest details.

### 2.1.6 DISC STORAGE

Store commercially prepared individual discs or cartridges of discs in sealed containers with an indicating desiccant and protect them from light. This is especially important for light-susceptible agents such as metronidazole, chloramphenicol and the quinolones. Store stocks as recommended by the manufacturer (-20°C to 8°C). Store working supplies of discs at <8°C in sealed containers with an indicating desiccant. Discard any discs on the expiry date shown on the side of the packaging. To prevent condensation forming on discs, allow them to warm to room temperature before opening containers. It is better to leave discs in sealed containers at room temperature during the day than repeatedly to transfer them from <8°C to room temperature. These requirements apply also to discs in dispensers.

### 2.1.7 DISC APPLICATION

Use cooled, flamed forceps or a disc dispenser to place discs on plates. Apply discs to plates within 15 minutes of inoculation, otherwise organisms may grow sufficiently to reduce zone sizes. Firmly apply discs to the inoculated agar surface to ensure even contact with the plate. To avoid unacceptable overlap of zones, there should be no more than six discs per 90 mm plate and no more than 12 discs per 150 mm plate. The agents begin to diffuse immediately on contact with the agar so do not relocate discs on plates.

### 2.1.8 INCUBATION

Incubate plates within 15 minutes of disc application otherwise agents may diffuse from discs before incubation commences, which increases zone sizes. Do not stack plates more than six high in the incubator, otherwise uneven heating may result in larger zones of inhibition. Larger stacks are acceptable if shown to have no effect on zone diameters. Incubation conditions are as follows:

Organisms	Incubation conditions
Enterobacteriaceae	35-37°C in air for 18-20 h
<i>Pseudomonas</i> species	35-37°C in air for 18-20 h
<i>S. maltophilia</i>	30°C in air for 18-20 h
Staphylococci (agents other than methicillin/oxacillin/cefoxitin)	35-37°C in air for 18-20 h
<i>S.aureus</i> using cefoxitin for detection of methicillin/oxacillin/cefoxitin resistance	35°C in air for 18-20 h
Staphylococci using methicillin/oxacillin to detect resistance	30°C in air for 24h
<i>M. catarrhalis</i>	35-37°C in air for 18-20 h
α-Haemolytic streptococci	35-37°C in 4-6% CO <sub>2</sub> in air for 18-20 h
β-Haemolytic streptococci	35-37°C in air for 18-20 h
Enterococci	35-37°C in air for 24 h
<i>N. meningitidis</i>	35-37°C in 4-6% CO <sub>2</sub> in air for 18-20 h
<i>S. pneumoniae</i>	35-37°C in 4-6% CO <sub>2</sub> in air for 18-20 h
<i>Haemophilus</i> species	35-37°C in 4-6% CO <sub>2</sub> in air for 18-20 h
<i>N. gonorrhoeae</i>	35-37°C in 4-6% CO <sub>2</sub> in air for 18-20 h
<i>P. multocida</i>	35-37°C in 4-6% CO <sub>2</sub> in air for 18-20 h

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Coryneform organisms	35-37°C in 4-6% CO <sub>2</sub> in air for 18-20 h
<i>Campylobacter</i> species	35-37°C in air for 18-20 h
<i>B. fragilis</i> , <i>B. thetaiotaomicron</i> , <i>C. perfringens</i>	35-37°C in 10% CO <sub>2</sub> / 10% H <sub>2</sub> / 80% N <sub>2</sub> for 18-20 h (anaerobic cabinet or jar)

It is essential to incubate enterococcal plates for at least 24h before reporting a strain as susceptible to vancomycin or teicoplanin as small colonies within zones may not be visible with shorter incubation. Any organism failing to give visible growth after overnight incubation must not be tested by disc diffusion as false susceptible reports may result. Such strains should be tested by an MIC method.

### 2.1.9 READING RESULTS

Plates containing media unsupplemented with blood may be read from the upper surface or from the underside of the plate through the medium. If the medium contains blood, read plates from the upper surface with the cover removed. Zones of inhibition should be circular. Exceptions to this may be due to faulty testing or to antagonism or synergy between adjacent agents. The growth should be semi-confluent (there should be no spaces between individual colonies and the growth should not be confluent). If the growth is either too heavy or too light, repeat the test. Use a ruler, vernier callipers or automated zone reader to measure diameters of zones of inhibition (mm). Alternatively, a template can be used to interpret susceptibility without measuring zones (see section 2.1.10).

Judge the zone edge by the naked eye. Ignore minute colonies at the edge of the zone, films of growth due to swarming of *Proteus* species, slight growth within sulphonamide or trimethoprim zones and zones of haemolysis. Subculture, identify and re-test any colonies growing within the zone of inhibition, if necessary. With staphylococci tested against methicillin/oxacillin/cefoxitin, and staphylococci and enterococci tested against vancomycin, any discernible growth within the zone of inhibition may indicate resistance.

### 2.1.10 INTERPRETATION

Zone diameters are interpreted by reference to the tables of breakpoints<sup>5</sup>. The most up to date version of the breakpoints is available from the BSAC web site<sup>6</sup>, which must be used in preference to the original printed version<sup>5</sup>. If templates (eg Appendix 3) are used to interpret tests, place the plate over the template and interpret the zones according to the zone diameter breakpoints marked on the template. With media containing blood, place the template (printed on transparent material) over the plate. A computer program for preparation of templates is available from the BSAC web site<sup>6</sup>.

### 2.1.11 QUALITY CONTROL<sup>6</sup>

Control strains, as appropriate, should be tested daily. Once it has been shown that control zones are consistently within range the frequency may be reduced to weekly testing. If control zones subsequently fall outside the acceptable range in more than 1 in 20 tests the frequency of testing should revert to daily until tests are again in range.

Control strains must be maintained under conditions which preserve strain characteristics<sup>28</sup>.

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Control organism	Either	Or
<i>Escherichia coli</i> <i>Escherichia coli</i>	NCTC 12241 (ATCC 25922) NCTC 11560 <sup>a</sup>	NCTC 10418
<i>S. aureus</i> <i>S. aureus</i>	NCTC 12981 (ATCC 25923) NCTC 12493 <sup>b</sup>	NCTC 6571
<i>S. pneumoniae</i>	NCTC 12977 <sup>c</sup> (ATCC 49619)	
<i>E. faecalis</i>	NCTC 12697 (ATCC 29212)	
<i>P. aeruginosa</i>	NCTC 12903 (ATCC 27853)	NCTC 10662
<i>H. influenzae</i> <i>H. influenzae</i>	NCTC 12699 (ATCC 49247) NCTC 12699 <sup>d</sup> (ATCC 49247)	NCTC 11931
<i>N. gonorrhoeae</i>	NCTC 12700 <sup>c</sup> (ATCC 49226)	
<i>P. multocida</i>	NCTC 8489	
<i>B. fragilis</i>	NCTC 9343 (ATCC 25285)	
<i>B. thetaiotaomicron</i>		ATCC 29741
<i>C. perfringens</i>	NCTC 8359 (ATCC 12915)	

<sup>a</sup>  $\beta$ -lactamase producing strain for testing  $\beta$ -lactamase inhibitor combinations

<sup>b</sup> MRSA strain for testing detection of methicillin resistance in *S. aureus*

<sup>c</sup> Low-level resistant to penicillin

<sup>d</sup> Resistant to beta-lactams (beta-lactamase negative)

Confirm that the zone of inhibition for the control strain falls within the acceptable range before interpreting the test. Control zone diameters that lie outside acceptable ranges in more than 1 out of 20 tests indicates that there is likely to be a problem with the method and possible sources of error must be investigated. If controls are out of range tests must be interpreted with caution and, depending on the cause of the error, it should be assessed whether to withhold results and/or repeat tests.

Recording of control zone sizes on a chart which is marked with limits makes abnormalities easy to see and gradual trends are more obvious.

Use a methicillin/oxacillin heteroresistant strain of *S. aureus* (NCTC 12493) with new batches of medium to confirm that resistance is detected (colonies should grow up to the disc).

Use a  $\beta$ -lactamase producing strain of *E. coli* (NCTC 11954 or NCTC 11560) to detect deterioration of the  $\beta$ -lactamase inhibitor in discs containing a combination of a  $\beta$ -lactamase inhibitor and a  $\beta$ -lactam agent. Zone sizes decrease as the  $\beta$ -lactamase inhibitor deteriorates.

## 2.2 BREAKPOINT METHOD

As with other techniques, the results of breakpoint tests are significantly influenced by methodology, which must be carefully controlled to obtain accurate and reproducible results. The method described is an agar breakpoint method based on the MIC method recommended by the BSAC<sup>7</sup> and the breakpoints used are the MIC breakpoints separating the different categories of interpretation as recommended by the BSAC<sup>29</sup> (see <http://www.BSAC.org.uk> for latest breakpoints).

### 2.2.1 MEDIUM CONTAINING ANTIMICROBIAL AGENTS

Use the media as described in section 2.1.1. Add antimicrobial agents to molten medium, cooled to 45°C, and mix well before plates are poured. Antimicrobial agents may be added as solutions made from pure antibiotic powders, or commercial freeze dried agents in vials.

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Tablets specifically prepared for breakpoint testing may also be used. Store and use commercial preparations as recommended by the manufacturers.

Final concentrations of antimicrobial agents in agar are as recommended by the BSAC for MIC breakpoints<sup>29</sup> (see <http://www.BSAC.org.uk> for latest breakpoints).

Plate drying, storage conditions and shelf life should be determined by media production departments and controlled as part of the media quality assurance programme. Note that the shelf life of particularly labile agents, such as imipenem and clavulanic acid combinations such as co-amoxiclav and ticarcillin-clavulanic acid, is very short and that plates containing these agents should be prepared on the day of use.

## **2.2.2 INOCULUM PREPARATION**

Prepare and standardise the inoculum by use of a barium sulphate 0.5 McFarland standard or latex equivalent as described for disc diffusion testing (see 2.1.3). Alternative methods may be used if they give equivalent results. Dilute the standardised suspension in sterile distilled water so that the inoculum delivered to the plates is 10<sup>4</sup> CFU/spot. Inocula standardised to the density of a 0.5 McFarland standard will require a 1:10 dilution if inoculator pins which deliver 1 µL are used. Check the inoculum for purity by subculture of suspensions to appropriate media.

## **2.2.3 INOCULATION OF TEST PLATES**

Transfer inocula to sterile inoculum wells of the replicating device. Take care to avoid cross contamination of wells. Do not completely expel micropipettes when filling wells as this may cause splashing. Use a multipoint inoculator with sterile inoculator pins to transfer inoculum to test plates. The number of test organisms per plate will depend on plate size and the format of the inoculator pins. Inoculate first a control plate without antibiotic followed by plates containing antimicrobial agents.

## **2.2.4 INCUBATION**

As soon as the spots of inoculum are dry invert the plates and incubate as for disc diffusion tests (see section 2.1.8).

## **2.2.5 READING OF RESULTS**

Examine purity plates and repeat the tests if cultures are mixed. Examine the control plate without antimicrobial agents to ensure that growth is adequate. Examine plates for presence or the absence of growth. Disregard single colonies or a faint haze produced by the inoculum.

## **2.2.6 INTERPRETATION OF RESULTS**

The organism is considered susceptible if there is no growth on the plate containing the agent and adequate growth on the control plate. The organism is considered resistant if it has grown on the plate containing antibiotic.

## **2.2.7 QUALITY CONTROL**

Effective daily control is difficult because MICs of agents for control strains used in other methods may be several two-fold dilutions away from the breakpoint concentrations and for most agents the daily control strains (see section 2.1.11) will detect only gross errors.

Batches of plates should ideally be controlled before use by inoculation with control strains which for each agent have MICs one dilution above and below breakpoints. However, there is no standard set of strains available to meet this requirement.

Consistency between successive batches can be tested by including a set of plates from each batch and comparing overall results. Significant changes in the overall pattern of routine results may also indicate problems.

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## 2.3 ETEST MICs

The Etest is a commercial MIC test and detailed instructions are given by the manufacturer for performance of the test.

### Notes:

Use the method as described by the manufacturer. The manufacturer indicates that for most organisms Iso-Sensitest agar is acceptable except for tests on carbapenems, oxacillin testing of staphylococci and glycopeptide testing of enterococci and staphylococci. See technical manuals (available from the supplier) for tests on anaerobes, meningococci, gonococci and haemophili.

The inoculum is standardised to the density of a McFarland standard recommended by the manufacturer for the particular organism-antimicrobial combination tested. Note that different inocula may be recommended for different organism-antimicrobial agent combinations. The standardised suspension is used undiluted (the method has been calibrated for these inocula). Use of a lighter inoculum may give lower MICs.

If the test is controlled by inclusion of a control organism on the same plate as the test, inoculate the test organism on half of the plate and the control organism on the other half of the plate, with a 2-3 mm gap between the two organisms. Take care to apply the strip on the centre of the line between the test and control strains. The test may be controlled using a separate plate, but this does not control the individual strip and requires use of an additional strip.

Read the MIC from the scale where the ellipse edge intersects the strip. Read at the point of inhibition of all growth, including hazes and isolated colonies, except where otherwise indicated by the manufacturer, eg 80% endpoints are used for bacteriostatic agents including sulphonamides, trimethoprim, linezolid, quinupristin/dalfopristin, tetracyclines, chloramphenicol, macrolides, azithromycin and clindamycin. When growth occurs along the entire strip and no inhibition is seen, report the MIC as greater than the highest value on the reading scale. When the ellipse is so large that the zone edge does not intersect the strip, report the MIC as less than the lowest reading on the scale.

MIC ranges for control strains are given by the manufacturer.

## 2.4 ANAEROBIC BACTERIA

Susceptibility testing for anaerobes is recommended in clinical situations where there is known resistance in a particular species, failure of the usual therapeutic regimen, when infection is severe or long term therapy is required. It is normally necessary to test isolates from brain abscess, endocarditis, osteomyelitis, joint infections, infection of prosthetic devices, vascular grafts and bacteraemia. Note that anaerobic infections may involve a mixed aerobic-anaerobic flora, and recommendations for treatment may need to cover both anaerobes and aerobic organisms.

A tentative disc diffusion method has been recommended by the BSAC<sup>6</sup> for testing rapidly growing anaerobes against metronidazole only. Resistance should be confirmed with an MIC method. Criteria for other agents are not currently available and an MIC method is recommended. An agar dilution breakpoint method<sup>30</sup> may be used but is not practicable for small numbers of tests. In view of the limited number of tests set up routinely on anaerobes, for those combinations where disc diffusion cannot be used the Etest MIC method as described by the manufacturer is recommended<sup>31</sup> (an extensive reference list is available from the supplier). Alternatively, strains should be sent to the Anaerobe Reference Unit.

### Method for disc diffusion testing<sup>6</sup>

Prepare ISA agar supplemented with 5% whole horse blood and 20 mg/L NAD (see section 2.1.2).

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Use colonies from a freshly plated culture. Suspend colonies in distilled water and adjust the density to that of a 0.5 McFarland standard. Dilute the suspension in distilled water, 1 in 100 for Gram-negative species and 1 in 10 for Gram-positive species.

Use a sterile swab to spread the inoculum evenly over the surface of the medium.

Incubate plates at 37°C for 18-24 h under strict anaerobic conditions. It is important to control anaerobiosis because pseudo-resistance to metronidazole may result from incubation in sub-optimal anaerobic conditions.

Tentative zone diameter breakpoints for interpretation are given in Appendix 4, together with MIC breakpoints<sup>6</sup>.

## 2.5 FUNGI

Antifungal drug susceptibility tests are markedly affected by test conditions<sup>32</sup> and, with azole compounds, partial inhibition of growth occurs over a wide range of concentrations of the drug, making it difficult to read endpoints<sup>33</sup>. Yeasts may be tested by disc diffusion methods for voriconazole and fluconazole<sup>34,35</sup>. Other methods for testing yeasts are the Etest<sup>36,37</sup> (an extensive reference list is available from the supplier) and Sensititre<sup>38</sup> systems, which must be set up according to the manufacturer's instructions, or the broth microdilution method<sup>39,40</sup>. Alternatively, isolates should be sent to the Mycology Reference Laboratory. In addition species with rare resistance and isolates of filamentous fungi should be sent to the Mycology Reference Laboratory for antimicrobial susceptibility testing.

## 2.6 OTHER ORGANISMS

### 2.6.1 HELICOBACTER PYLORI

Methods for susceptibility testing of *H. pylori* have not been standardised. There are widely differing views on appropriate methodologies and interpretation of tests. In particular, results with metronidazole are dependent on whether incubation conditions are microaerobic, or anaerobic followed by microaerobic<sup>41,42</sup>. Metronidazole resistant sub-populations may also be a significant cause of variation in test results<sup>43</sup> that can be significant for treatment outcomes and may be missed by methods other than the Etest. Similar observations have been made with clarithromycin resistant strains<sup>44</sup>. A European multicentre study has, however, found that there are also limitations to the Etest with metronidazole<sup>45</sup>. Any method must therefore be considered tentative and the following are recommended:

#### **Etest method proposed by the European *H. pylori* Study Group<sup>45</sup>**

Resuspend the test strain (48-72 h culture) in peptone water to a density equivalent to a 4 McFarland standard.

Use a sterile swab to spread the inoculum evenly on a plate of Mueller-Hinton agar with 10% whole horse blood.

Apply Etest strips to the medium after the inoculum has dried. Use one Etest strip per 9 cm plate.

Incubate in microaerobic conditions at 35°C - 37°C for 48 h.

It is important to minimise exposure of isolates to aerobic conditions. Laboratories should aim to remove plates from microaerobic conditions, inoculate broths, plates and reincubate within a maximum of 45 minutes.

Read MICs at the point of intersection of the zone of inhibition with the Etest strip. Colonies may be small and translucent. Resistant mutants may grow as isolated colonies within zones. It may be useful to tilt the plate in the light and use a magnifying glass when reading MICs.

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Tentative MICs for the control strain, *H. pylori* NCTC 11637 (ATCC 43504) are given in Appendix 5a, together with criteria for interpretation of results.

#### **Disc diffusion method proposed by the Health Protection Agency *H. pylori* Study Group<sup>46</sup>**

Resuspend the test strain (48-72 h culture) to a density equivalent to a 4 McFarland standard.

Use a sterile swab to spread the inoculum evenly on a plate of Mueller-Hinton agar with 5-10% whole horse or sheep blood (or Columbia agar with 5-10% blood).

After the inoculum has dried apply metronidazole 5 µg and clarithromycin 2 µg discs to the medium.

Incubate in microaerobic conditions at 35°C - 37°C for 48 h.

Include a metronidazole susceptible control strain, eg NCTC 12822.

It is important to minimise exposure of isolates to aerobic conditions. Laboratories should aim to remove plates from microaerophilic conditions, inoculate broths and plates, and reincubate within a maximum of 45 minutes.

Colonies may be small and translucent. Resistant mutants may grow as isolated colonies within zones. MICs should be determined for strains which appear intermediate in susceptibility to metronidazole.

Criteria for interpretation of zones, are given in Appendix 5b.

#### **2.6.2 CAMPYLOBACTER SPECIES**

There is considerable variability in methods used for *Campylobacter* species. The BSAC have made recommendations for a disc diffusion method<sup>47</sup> for testing erythromycin, ciprofloxacin and nalidixic acid (used to detect quinolone resistance) only as tests on other agents by disc diffusion are unreliable. The method is as follows:

Suspend colonies in sterile distilled water to a density equivalent to a 0.5 McFarland standard.

Use a sterile swab to spread the inoculum evenly on a plate of Iso-Sensitest agar with 5% whole horse blood.

After the inoculum has dried apply discs with contents as in Appendix 6.

Incubate in microaerobic conditions at 42°C (35°C - 37°C for *C. fetus*) for 18-24 h.

Tentative interpretative criteria are given in Appendix 6.

Alternatively the Etest method may be used according to the manufacturer's instructions (reference list available from the manufacturer).

#### **2.6.3 LISTERIA SPECIES**

An Etest method according to the manufacturer's instructions is recommended, with breakpoints as defined by BSAC for streptococci<sup>29</sup> (except for benzyl penicillin where breakpoint is as for ampicillin, S ≤ 1 mg/L, R ≥ 2 mg/L).

#### **2.6.4 LEGIONELLA SPECIES**

Disc diffusion methods are unsuitable because *Legionella* are slow growing. An agar dilution MIC method on buffered yeast extract agar with 5% water-lysed horse blood is recommended<sup>47,48</sup>. There are no validated MIC breakpoints for *Legionella*, but if MICs are within the ranges for the wild-type distribution<sup>47</sup> the isolates can be considered to be clinically susceptible.

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### 2.6.5 *STENOTROPHOMONAS MALTOPHILIA*

Susceptibility tests for aminoglycosides are affected by temperature. Tests on  $\beta$ -lactam agents are affected by the medium used. Most  $\beta$ -lactams have poor activity against *S. maltophilia* and isolates may appear falsely susceptible in diffusion tests on ISA (they appear more resistant on Muller-Hinton agar). Susceptibility testing is not recommended except for co-trimoxazole (see [www.bsac.org.uk](http://www.bsac.org.uk) BSAC Standardized Susceptibility Testing Method, Additional Methodology, *Stenotrophomonas maltophilia*). The following test conditions are recommended: Iso-Sensitest agar, semi-confluent growth, 25  $\mu$ g co-trimoxazole disc, incubation at 30°C for 18-20 h, susceptible =  $\geq$  20 mm, resistant  $\leq$  19 mm.

### 2.6.6 CYSTIC FIBROSIS ISOLATES OF *PSEUDOMONAS AERUGINOSA*

Testing susceptibility of *P. aeruginosa* isolates from cystic fibrosis patients is problematic. This is because different colonies from the same specimen may give widely different susceptibilities to the same agent and because the in-vitro/in vivo correlation is poor. It is more practical for routine purposes to test mixed multiple colonies from the growth on primary isolation plates and to report the susceptibility of the most resistant<sup>49</sup>, although there is little evidence that this approach, or results with any other susceptibility test method, correlates with clinical outcome<sup>50</sup>. Further tests with single types may be considered appropriate if organisms are resistant.

### 2.6.7 VANCOMYCIN INTERMEDIATE *S. AUREUS* (VISA)

*S. aureus* isolates with vancomycin MICs of 8 mg/L (termed VISA or GISA – glycopeptide intermediate *S. aureus*) appear to be rare, although there have been reports from Japan<sup>51</sup>, the United States<sup>52</sup>, France<sup>53</sup> and the UK<sup>54</sup>. Strains which are heterogeneous in susceptibility to vancomycin (termed hetero-VISA) and which have MICs  $\leq$  4 mg/L by conventional MIC methods are much more common although the clinical significance of such isolates is not clear. Vancomycin intermediate *S. aureus* cannot be detected by disc diffusion methods<sup>55</sup>. In the BSAC disc diffusion method<sup>5</sup> it is noted that population analysis profile (PAP) studies are the most reliable method for detecting heterogeneous resistance to vancomycin. However, such methods are not practical for routine use and it is suggested that if reduced susceptibility to vancomycin is suspected on clinical grounds, the organism should be sent to a reference laboratory for confirmation. Six clinical isolates of *S. aureus* resistant to glycopeptides have been reported in the USA<sup>56-58</sup> (see CDC website [www.cdc.gov](http://www.cdc.gov) for latest details). The isolates carried the *vanA* gene cluster from enterococci, and vancomycin resistance was detectable by disc diffusion methods, with growth inside a zone of inhibition, but two were not detected by Vitek automated methods<sup>58,59</sup>.

A screening test recommended by CDC<sup>60</sup> for VISA involves spotting 10  $\mu$ L 0.5 McFarland suspension bacteria ( $10^6$  CFU) on BHI agar containing 6 mg/L vancomycin. Plates are incubated at 35°C for 48 h and examined for colonies at 24 h and 48 h. This method will detect resistant strains and VISA but not hetero-VISA strains. A modified Etest method (the “macro” Etest) may be used to screen for both GISA and hetero-GISA strains<sup>61</sup>. This method requires use of BHI agar, an inoculum adjusted to a 2.0 McFarland standard, incubation at 35°C for 48 h, and specific interpretative criteria – vancomycin and teicoplanin MICs both  $\geq$  8 mg/L or teicoplanin MIC alone  $\geq$  12 mg/L are indicative of reduced susceptibility to glycopeptides. Any suspect isolates should be confirmed by the PAP method or sent to the Antibiotic Resistance Monitoring and Reference Laboratory, Colindale for confirmation.

## 2.7 TESTING FOR $\beta$ -LACTAMASE<sup>62</sup>

Acidimetric tests are reliable for detecting  $\beta$ -lactamases in staphylococci, *H. influenzae* and *N. gonorrhoeae*. Nitrocefin-based tests are reliable for tests on *H. influenzae*, *N. gonorrhoeae* and *M. catarrhalis*, but are not reliable for staphylococci. Routine testing for staphylococcal  $\beta$ -lactamases is not justified.

Use commercially produced  $\beta$ -lactamase tests according to the manufacturer’s instructions.

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Use known positive and negative control strains as follows:

Control organism	$\beta$ -lactamase negative control	$\beta$ -lactamase positive control
<i>H. influenzae</i>	NCTC 12699 (ATCC 49247)	NCTC 11315
<i>N. gonorrhoeae</i>	NCTC 12700 (ATCC 49226)	NCTC 11148

A positive  $\beta$ -lactamase test predicts resistance to penicillin, ampicillin and amoxicillin among *Haemophilus* species, *N. gonorrhoeae* and *M. catarrhalis*. It also predicts resistance to penicillin as well as acylamino-, carboxy- and ureido-penicillins among staphylococci and enterococci. A negative  $\beta$ -lactamase test does not rule out resistance due to other mechanisms. Do not test other aerobic Gram-negative rods because the results will not predict susceptibility to  $\beta$ -lactams used for therapy.

Extended-spectrum  $\beta$ -lactamases (ESBLs) in Enterobacteriaceae are plasmid mediated enzymes which have an extended substrate range including extended spectrum cephalosporins (eg ceftazidime, cefotaxime) and monobactams (eg aztreonam)<sup>63</sup>. Most ESBLs historically were mutants of the TEM and SHV enzymes (see <http://www.lahey.org/studies>), but CTX-M enzymes<sup>64</sup> are now more common in the UK (see QSOP 51 - Laboratory detection and reporting of bacteria with extended spectrum  $\beta$ -lactamases). There are also types rare in the UK such as VEB and PER. ESBLs have been found worldwide and have been detected in most Enterobacteriaceae, most often in *Klebsiella pneumoniae* but increasingly also in *E.coli*. Epidemics of infection with single strains of *K. pneumoniae* have been widely described, but epidemiology may be complex as there may also be spread of plasmids carrying ESBLs among different strains<sup>65</sup>. CTX-M ESBLs are often in *E. coli* from patients in hospitals and the community, but some patients have had no known contact with hospitals.

Not all Enterobacteriaceae resistant to third generation cephalosporins are ESBL producers. Resistance may be due to hyperproduced chromosomal AmpC  $\beta$ -lactamases especially in *Enterobacter* species, plasmid-mediated AmpC  $\beta$ -lactamases in *Klebsiella* species, and *E. coli* and hyperproduced K1 chromosomal  $\beta$ -lactamases in *K. oxytoca*.

Advice on ESBL detection is presented separately (see QSOP 51 - Laboratory detection and reporting of bacteria with extended spectrum  $\beta$ -lactamases).

The double disc synergy test is practical for routine use<sup>62,66</sup>. Place discs containing ceftazidime 30  $\mu$ g, cefotaxime 30  $\mu$ g or aztreonam 30  $\mu$ g at a distance of 30 mm (centre to centre) around a disc of co-amoxiclav 30  $\mu$ g. ESBL-producers show enhanced zones between the co-amoxiclav and one or more of the other discs. The distance between discs is critical and false negatives may occur with *Proteus mirabilis* which give large zones.

Etest ESBL strips may be used<sup>67</sup>. They consist of a strip with a ceftazidime or cefotaxime gradient at each end, one of the gradients also containing clavulanic acid. A difference  $\geq 8$  in the ratio of ceftazidime or cefotaxime MICs with and without clavulanic acid indicates ESBL production. Follow the manufacturer's instructions when setting up this test.

Combined disc methods are also commercially available. These methods depend on differences in zone diameter between discs containing ceftazidime<sup>68</sup>, cefotaxime<sup>69</sup> or cefpodoxime<sup>69</sup> and discs containing the same agents with clavulanic acid. Follow the manufacturer's instructions when setting up these tests.

Quality control of cefpodoxime, cefotaxime and ceftazidime discs used in screening should be as required for standard BSAC methods. Positive controls should also be used with ESBL confirmatory tests and three ESBL-positive *E. coli* strains are recommended: CTX-M-15 (cefotaximase) NCTC 13353; TEM-3 (broad-spectrum) NCTC 13351; TEM-10 (ceftazidimase)

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NCTC 13352 (see QSOP 51 - Laboratory detection and reporting of bacteria with extended spectrum  $\beta$ -lactamases).

## **3 REFERRAL TO REFERENCE LABORATORY**

### **3.1 REFERRAL TO REFERENCE LABORATORIES**

Isolates associated with outbreaks and where epidemiologically indicated.

Organisms with unusual (see Appendix 7) or unexpected resistance, and whenever there is a laboratory or clinical problem or anomaly that requires elucidation.

Filamentous fungi for susceptibility testing:

Mycology Reference Laboratory  
SouthWest HPA Laboratory,  
Myrtle Road,  
Kingsdown,  
Bristol BS2 8EL  
Tel: 0117 9285028 / 0117 9291326

Anaerobic isolates for susceptibility testing:

Anaerobe Reference Unit  
NPHS Microbiology Cardiff  
University Hospital of Wales  
Heath Park  
Cardiff CF14 4XW

Tel: 0292074 2171

## **4 REPORTING PROCEDURE**

### **4.1 SUSCEPTIBILITY TESTING**

Report:

Susceptible, Intermediate (use limited to a few specific combinations) or Resistant.

Release susceptibility results as clinically indicated according to local protocol.

Also, report results of supplementary investigations.

### **4.2 CULTURE REPORTING TIME**

Clinically urgent culture results to be telephoned or sent electronically.

Written report: 16 – 72 h stating, if appropriate, that a further report will be issued.

Supplementary investigations additional tests results from reference laboratory.

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## 5 REPORTING TO THE HPA (LOCAL AND REGIONAL SERVICES AND CDSC CENTRE FOR INFECTIONS)<sup>70</sup>

Refer to the following:

Health Protection Agency publications:

"Hospital infection control: Guidance on the control of infection in hospitals"

Current guidelines on CDSC and COSURV reporting

Local guidelines

Report all isolates of the following:

Isolates associated with emerging resistance (Appendix 7)

## 6 ACKNOWLEDGEMENTS AND CONTACTS

This National Standard Method has been developed, reviewed and revised by the Standard Methods Working Group for Clinical Bacteriology ([http://www.hpa-standardmethods.org.uk/wg\\_bacteriology.asp](http://www.hpa-standardmethods.org.uk/wg_bacteriology.asp)). The contributions of many individuals in clinical bacteriology laboratories and specialist organisations who have provided information and comment during the development of this document, and final editing by the Medical Editor, are acknowledged.

The National Standard Methods are issued by Standards Unit, Department for Evaluations, Standards and Training, Centre for Infections, Health Protection Agency, London.

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## APPENDIX 1: DISC CONTENTS ( $\mu\text{G}$ ) FOR DISC DIFFUSION TESTS ON NON-FASTIDIOUS ORGANISMS (SEE BSAC WEBSITE FOR RECENT ADDITIONS<sup>6</sup>)

Agent	Enterobacteriaceae <i>Acinetobacter</i> species	<i>Pseudomonas</i> species	<i>Staphylococcus</i> species	<i>Enterococcus</i> species	UTI Gram negative rods	UTI Gram positive cocci
Amikacin	30	30	30			
Amoxicillin	10				25	
Ampicillin	10			10	25	25
Azithromycin			15	15		
Aztreonam	30	30				
Carbenicillin		100				
Cefaclor	30					
Cefamandole	30					
Cefepime	30					
Cefixime	5					
Cefoperazone	30					
Cefotetan	30					
Cefotaxime	30	30				
Cefoxitin	30		10			
Cefpodoxime	10					
Cefpirome	20	20				
Ceftazidime	30	30				
Ceftibuten	10					
Ceftizoxime	30					
Ceftriaxone	30	30				
Cefuroxime	30					
Cephalothin	30					
Cephalexin					30	30
Cephradine	30					
Chloramphenicol	30		10			
Ciprofloxacin	1	1 or 5	1		1	1 or 5
Clarithromycin			2			

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Agent	Enterobacteriaceae <i>Acinetobacter</i> species	<i>Pseudomonas</i> species	2 <i>Staphylococcus</i> species	<i>Enterococcus</i> species	UTI Gram negative rods	UTI Gram positive cocci
Clindamycin			2			
Co-amoxiclav	30		3		30	30
Colistin	25	25				
Co-trimoxazole	25	25	25			
Doxycycline	30					
Ertapenem	10					
Erythromycin			5			
Fosfomycin/glucose-6-phosphate					200/50	200/50
Fusidic acid			10			
Gatifloxacin	2	2	2			
Gemifloxacin	1	5	1			
Gentamicin	10	10	10	200		
Imipenem	10	10		10		
Levofloxacin	1	5				
Linezolid			10	10		
Mecillinam					10	50
Meropenem	10	10		10		
Methicillin			5			
Mezlocillin	75					
Moxifloxacin	1	5	1			
Mupirocin			5 or 20			
Nalidixic acid					30	30
Neomycin			10			
Netilmicin		30				
Nitrofurantoin					200	200
Norfloxacin					2	2
Ofloxacin	5		5			
Oxacillin			1			
Penicillin			1U			
Piperacillin	75	75				
Piperacillin/tazobactam	75/10	75/10				
Quinupristin/dalfopristin			15	15		

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Rifampicin			2			
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Agent	Enterobacteriaceae <i>Acinetobacter</i> species	<i>Pseudomonas</i> species	<i>Staphylococcus</i> species	<i>Enterococcus</i> species	UTI Gram negative rods	UTI Gram positive cocci
Streptomycin	10					
Sulphamethoxazole	100					
Teicoplanin			30	30		
Telithromycin			15			
Tetracycline			10			
Ticarcillin		75				
Timentin	85	85				
Tobramycin	10	10	10			
Trimethoprim	2.5		5		2.5	2.5
Vancomycin			5	5		

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## APPENDIX 2: DISC CONTENTS ( $\mu\text{G}$ ) FOR DISC DIFFUSION TESTS ON FASTIDIOUS ORGANISMS (SEE BSAC WEBSITE FOR RECENT ADDITIONS<sup>6</sup>)

Agent	<i>Streptococcus pneumoniae</i>	Haemolytic streptococci	<i>Moraxella catarrhalis</i>	<i>Neisseria gonorrhoeae</i>	<i>Neisseria meningitidis</i>	<i>Haemophilus influenzae</i>
Amoxicillin						2
Ampicillin			2			2
Azithromycin	15	15		15		15
Cefaclor	30		30			30
Cefixime	5	5		5		
Cefotaxime	5	5		5	5	5
Cefpodoxime	1					
Ceftazidime						30
Ceftibuten	10					
Ceftizoxime	30					
Ceftriaxone	30			5		30
Cefuroxime	5		5	5		5
Cephalothin		30				
Cephadroxil	30	30				
Cephalexin	30	30				
Chloramphenicol	10		10		10	10
Ciprofloxacin	1		1	1	1	1
Clarithromycin	2	2	2			5
Co-amoxiclav			3			2/1
Co-trimoxazole	25	25	25			25
Ertapenem	10		10			10
Erythromycin	5	5	5	5	5	5
Gatifloxacin	2		2			2
Gemifloxacin	1		1			1
Imipenem	10					10
Levofloxacin	1		1			1
Linezolid	10	10	10			

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<b>Agent</b>	<i>Streptococcus pneumoniae</i>	Haemolytic streptococci	<i>Moraxella catarrhalis</i>	<i>Neisseria gonorrhoeae</i>	<i>Neisseria meningitidis</i>	<i>Haemophilus influenzae</i>
Meropenem	10					10
Moxifloxacin	1		1			1
Nalidixic acid				30		30
Ofloxacin	5		5			5
Oxacillin	1					
Penicillin		1U		1U	1U	
Quinupristin/dalfopristin	15					
Rifampicin	5			2	2	
Spectinomycin				25		
Telithromycin	15		15			15
Tetracycline	10	10	10	10	10	10
Trimethoprim						2.5
Vancomycin	5					

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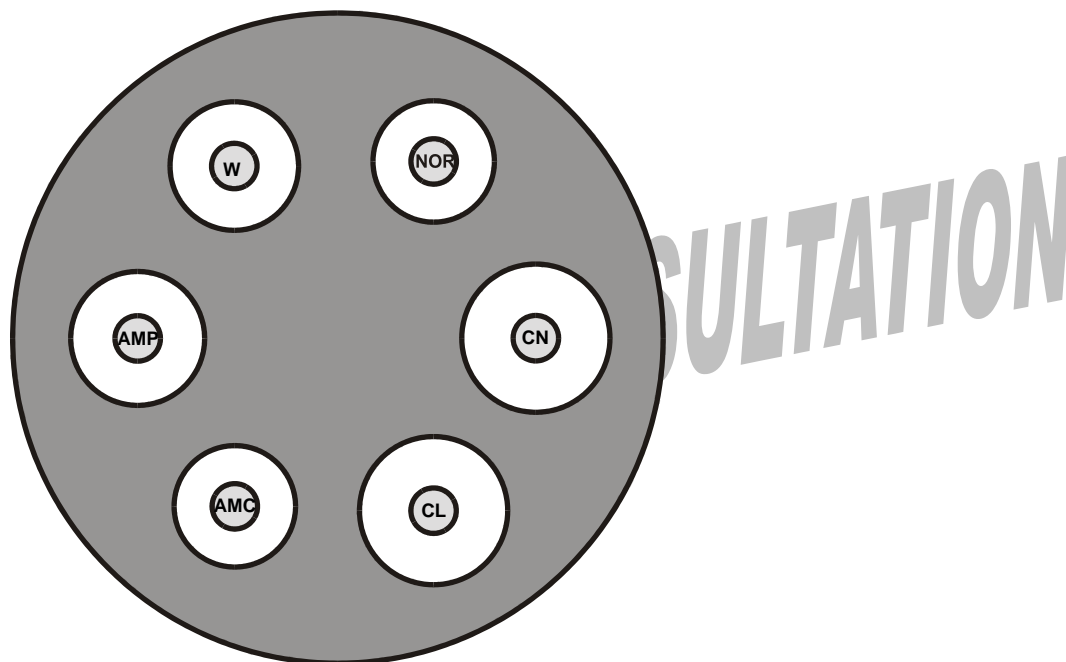
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## APPENDIX 3: EXAMPLE OF A TEMPLATE FOR INTERPRETATION OF ZONE DIAMETERS



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## APPENDIX 4: MIC AND ZONE DIAMETER BREAKPOINTS FOR ANAEROBIC ORGANISMS

Antibiotic	MIC breakpoint (mg/L)		Zone diameter breakpoints (mm)	
	R>	S≤	R≤	S≥
Cefoxitin	16	16	-	-
Chloramphenicol	8	8	-	-
Co-amoxiclav	4/2	4/2	-	-
Clindamycin	2	2	-	-
Imipenem	4	4	-	-
Metronidazole	8	8	17	18
Penicillin	1	1	-	-
Piperacillin/tazobactam	32/4	32/4	-	-
Tetracycline	4	4	-	-

## APPENDIX 5A: TENTATIVE MIC BREAKPOINTS FOR *HELICOBACTER PYLORI*

Antibiotic	MIC breakpoint (mg/L)		Acceptable MIC range for <i>Helicobacter pylori</i> ATCC 43504
	R >	S ≤	
Amoxicillin	1	1	0.015-0.125
Clarithromycin	1	1	0.015-0.125
Tetracycline	2	2	0.125-1
Metronidazole	4	4	64-256

## APPENDIX 5B: INTERPRETATION OF ZONE DIAMETERS FOR *HELICOBACTER PYLORI*

Antibiotic	Zone diameter breakpoint (mm) on Mueller-Hinton agar			Zone diameter breakpoint (mm) on Columbia agar	
	R	I	S	R	S
Clarithromycin 2µg	No zone	-	Any zone	No zone	Any zone
Metronidazole 5µg	<16	16-21 <sup>a</sup>	>21	<10	≥ 10

<sup>a</sup> Determine MIC for intermediate strains

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## APPENDIX 6: MIC AND ZONE DIAMETER BREAKPOINTS FOR *CAMPYLOBACTER* SPECIES

Antibiotic	MIC breakpoint (mg/L)		Disc content (µg)	Interpretation of zone diameter (mm)	
	R>	S≤		R	S
Erythromycin	0.5	0.5	5	≤19	≥20
Ciprofloxacin <sup>a</sup>	1	0.5	1	≤17	≥18
Nalidixic acid <sup>a</sup>	-	-	30	≤15	≥16

<sup>a</sup> Quinolone resistance is most reliably detected with nalidixic acid discs

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## APPENDIX 7: EXCEPTIONAL RESISTANCES

These unusual resistances should be checked locally and if confirmed the strains should be sent to the appropriate reference laboratory.

Organism	Resistances requiring referral to a reference laboratory	Reference Laboratory
<i>S. aureus</i>	Vancomycin Teicoplanin Linezolid Quinupristin/dalfopristin Daptomycin Tigecycline	ARMRL, Colindale
Coagulase-negative staphylococci	Vancomycin Linezolid Quinupristin/dalfopristin Daptomycin Tigecycline	ARMRL, Colindale
JK coryneforms	Vancomycin Teicoplanin Linezolid Quinupristin/dalfopristin	ARMRL, Colindale
<i>S. pneumoniae</i>	Meropenem Vancomycin Teicoplanin Linezolid Quinupristin/dalfopristin Tigecycline	ARMRL, Colindale
Group A, B, C, G $\beta$ -haemolytic streptococci	Penicillin Vancomycin Teicoplanin Linezolid Quinupristin/dalfopristin Daptomycin Tigecycline	ARMRL, Colindale
Enterococci	Both ampicillin and quinupristin/dalfopristin Linezolid Teicoplanin when vancomycin susceptible Tigecycline	ARMRL, Colindale
Enterobacteriaceae (eg. <i>Enterobacter</i> , <i>Escherichia</i> , <i>Citrobacter</i> , <i>Serratia</i> , <i>Proteus</i> , <i>Providencia</i> , <i>Klebsiella</i> , <i>Morganella</i> )	Meropenem Imipenem (except with <i>Proteus</i> species) Ertapenem	ARMRL, Colindale
<i>Salmonella</i> , <i>Shigella</i>	imipenem, ertapenem, meropenem	Laboratory of Enteric Pathogens, Colindale
<i>H. influenzae</i>	Third-generation cephalosporins (eg. cefotaxime, cefpodoxime, ceftazidime, ceftriaxone) Carbapenems (imipenem, meropenem) Tigecycline	ARMRL, Colindale
<i>M. catarrhalis</i>	Ciprofloxacin Third-generation cephalosporins (eg. cefotaxime, cefpodoxime, ceftazidime, ceftriaxone) Tigecycline	ARMRL, Colindale
<i>N. meningitidis</i>	Penicillin (high level) Ciprofloxacin	Meningococcal Reference Unit,

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		Manchester
<i>N. gonorrhoeae</i>	Third-generation cephalosporins (eg. cefotaxime, cefpodoxime, ceftazidime, ceftriaxone) and erythromycin	STRL, Colindale
<i>Acinetobacter</i> species	Colistin Carbapenems (eg. imipenem, meropenem)	ARMRL, Colindale
<i>P. aeruginosa</i>	Colistin	ARMRL, Colindale
All anaerobes	Metronidazole Carbapenems	Anaerobe Reference Unit, National Public Health Service Wales, Cardiff

These unusual resistances should be checked locally and if confirmed the strains should be sent to the appropriate reference laboratory.

<i>Bacteroides</i> species	Metronidazole Co-amoxiclav Carbapenems (eg. imipenem, meropenem)	Anaerobe Reference Laboratory, National Public Health Service Wales, Cardiff
<i>C. difficile</i>	Metronidazole Vancomycin	Anaerobe Reference Laboratory, National Public Health Service Wales, Cardiff

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