

Recommendations for susceptibility tests on fastidious organisms and those requiring special handling

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Fastidious organisms present problems in antimicrobial susceptibility testing related to particular cultural requirements or slow growth. Methods for commonly isolated fastidious organisms, including haemolytic streptococci, *Neisseria* spp. and *Haemophilus* spp., are included in the description of the standardized disc diffusion method. However, some further information is given here for *Haemophilus influenzae* and *Neisseria* spp. Other less commonly isolated fastidious organisms are detailed and modifications of the disc diffusion method are given for *Campylobacter* spp. and rapidly growing anaerobes. For those organisms where disc diffusion methodology is not recommended, *Brucella* spp., *Helicobacter pylori* and *Legionella* spp., MIC determinations are often needed, although disc diffusion test results may be useful as screens for resistance. Where disc diffusion can be used it is important to use the correct medium and to include appropriate controls. Methodology is also given for testing isolates of *Stenotrophomonas maltophilia*, which are affected by both medium and temperature.

Introduction

Some organisms present problems in susceptibility testing because of their growth requirements or because results are particularly affected by test conditions. Methods for testing commonly isolated aerobic fastidious organisms are given in the BSAC standardized disc diffusion method,¹ but some are referred to here to emphasize the difficulties in testing the organisms and to give further information. Although interpretative criteria for disc diffusion have not been published for some rapidly growing species that are isolated infrequently, disc diffusion susceptibility tests can be performed as a screen for high-level resistance or for presumptive susceptibility, but resistance must be confirmed by MIC determinations. The dynamics of disc diffusion methods make them unsuitable for susceptibility testing of slow-growing species and these should be tested by MIC method. The Etest (Cambridge Diagnostic Services, Cambridge, UK) is a technically straightforward method for determining MICs and is suitable for routine use in clinical laboratories. It is important to follow the techniques specified for each species in order to achieve the correct results.

Haemophilus influenzae

Interpretative criteria for zone diameters for *H. influenzae* are given in the standardized disc diffusion method.¹ Testing of *H. influenzae* is affected by medium, incubation atmosphere and inoculum size. The required medium is Iso-Sensitest agar supplemented with 5% defibrinated horse blood and 20 mg/L NAD. It is important that poured plates are not stored longer than the shelf-life determined by local media production facilities, as NAD may deteriorate and the isolates will grow poorly. The use of lysed horse blood or chocolate blood for susceptibility testing is not acceptable, as results for some antibiotics will be adversely affected. Zone diameter breakpoints, and acceptable zone diameter ranges for control strains of *H. influenzae* (and *Streptococcus pneumoniae*) have been determined after incubation in 4-6% CO₂ and are not applicable to tests incubated in air without added CO₂. If the inoculum is too heavy the tests may be misinterpreted as indicating resistance. β -Lactams are the most likely to be affected but zone diameters for most drugs will be reduced in size if the inoculum is too heavy. Tests on control strains must be set up daily to test the performance of the method, including the medium.

There has been a recent increase in the isolation rate of *H. influenzae* that appear resistant to cefuroxime by disc diffusion testing. These isolates have slightly increased MICs and may have decreased affinity of PBPs 3A and 3B or decreased outer-membrane permeability.²

Neisseria gonorrhoeae

Interpretative criteria for zone diameters for *N. gonorrhoeae* are given in the standardized disc diffusion method.¹ As for *H. influenzae*, the poured plates must not be stored longer than the locally determined shelf-life and control strains must be tested daily to ensure that growth is adequate. Zone diameters for susceptible isolates are often large and it is preferable to test only four discs per plate to reduce overlapping of zones.

The antimicrobials commonly used for treating gonorrhoea in the UK are a penicillin or a fluoroquinolone, usually ciprofloxacin, and susceptibility to these drugs should be tested routinely (testing with nalidixic acid is the most reliable method for detecting quinolone resistance in *N. gonorrhoeae*). Second line drugs for treatment of resistant isolates are spectinomycin and ceftriaxone. Isolates with chromosomally-encoded penicillin resistance (low-level) have slightly reduced zones of inhibition with ceftriaxone but remain susceptible. As resistance to ceftriaxone has not been described, the recommended zone breakpoint of ≥ 35 mm and MIC breakpoint of ≤ 0.25 mg/L for strains susceptible to this drug are tentative. Results for isolates with reduced zones around ceftriaxone discs should be confirmed by MIC determinations. Although tetracycline is not often used for the treatment of gonorrhoea, resistance is not uncommon. Resistance to tetracycline is usually easy to detect. High-level plasmid-mediated resistance is indicated by no zone around a 10 μ g tetracycline disc whereas susceptible isolates have large zones ≥ 27 mm. Some isolates have chromosomally encoded resistance, which confers low-level resistance, and these isolates will have reduced zone sizes. These isolates often carry other chromosomal resistances, particularly to penicillin.³

Neisseria meningitidis

Interpretative criteria for zone diameters for *N. meningitidis* are given in the standardized disc diffusion method.¹ *N. meningitidis* is a Hazard Group 2+ pathogen. Consequently suspension and dilution of organisms and inoculation of plates for susceptibility tests on *N. meningitidis* must be carried out in a Class1 safety cabinet. Reduced susceptibility to penicillin in *N. meningitidis* has

been described.⁴ If penicillin zone diameters are <20 mm the susceptibility should be confirmed by Etest. MIC determinations in place of disc diffusion tests for susceptibility testing of *N. meningitidis* should be considered as there are few isolates, but they are important, and there is little evidence that disc diffusion tests are reliable.

Anaerobes

The dynamics of disc diffusion technique, in which the size of the zone is related to a critical concentration of antimicrobial agent, a critical population of organisms and a critical time, makes it an unsuitable method for testing slow-growing organisms. However, anaerobes that grow well within 24 h can be tested by disc diffusion as a screen for resistance. The medium of choice for susceptibility testing of anaerobic bacteria is Wilkins-Chalgren agar supplanted with 5% horse blood. This medium adequately supports the growth of most anaerobic bacteria and has no adverse effect on the activity of antimicrobial agents.

- (i) Select colonies from a freshly plated culture. Suspend colonies in sterile distilled water and adjust the density to that of a 0.5 McFarland standard.
- (ii) Dilute the suspension in sterile distilled water, 1 in 100 for Gram-negative species and 1 in 10 for Gram-positive species.
- (iii) Use a sterile swab to inoculate evenly the surface of the medium.
- (iv) Incubate plates for 18-24 h at 37°C under strict anaerobic conditions.
- (v) Interpret zone diameters according to the tentative breakpoints given in Table I.

Resistance must be confirmed by MIC determinations. There are insufficient data available to recommend zone diameter breakpoints for other agents at this time.

There are particular problems when interpreting results for combinations containing β -lactamase inhibitors, as β -lactamase inhibitors have *in vitro* activity alone against many anaerobic species.

The BSAC Working Party recommendation for slow-growing anaerobic organisms is to test for susceptibility by MIC determinations. The medium recommended for the Etest by the manufacturer is Wilkins-Chalgren with 5% sheep blood. Sheep blood is not often used in the UK and horse blood can be substituted, but results for control organisms may be slightly different (follow Etest technical guide instructions).

Tests for complete anaerobiosis, such as anaerobic indicator BR55 (Oxoid, Basingstoke, UK) or lack of growth of *Pseudomonas aeruginosa* on Simmonds citrate agar, are essential when performing any susceptibility tests on anaerobes. Metronidazole and clindamycin are particularly affected by the presence of any oxygen, which will result in raised MICs or reduced zone sizes.

Helicobacter pylori

Disc diffusion methods are not suitable for testing *H. pylori* as this species is slow growing and results may not be accurate. The recommended method of susceptibility testing is Etest (follow Etest technical guide instructions).

- (i) Suspend colonies from a 2-3 day culture on a blood agar plate in sterile distilled water and adjust the density to equal a McFarland 3 standard.
- (ii) Use a swab dipped in the suspension to inoculate evenly the entire surface of the plate. The medium of choice is Mueller Hinton agar or Wilkins-Chalgren agar with 5-10% horse blood.
- (iii) Allow the plate to dry and apply Etest strip.
- (iv) Incubate at 35°C in microaerophilic conditions for 3-5 days.
- (v) Read the MIC at the point of complete inhibition of all growth, including hazes and isolated colonies. Tentative interpretative criteria for MICs are given in Table II.

Campylobacter spp.

The most commonly isolated *Campylobacter* spp. are those associated with gastrointestinal infection, *Campylobacter coli* and *Campylobacter jejuni*. Most *Campylobacter* spp. require a microaerophilic atmosphere for growth. Susceptibility tests for *Campylobacter* spp. are not standardized and therefore there is some variability in the susceptibility data reported in the literature. However, disc diffusion methods are suitable for detecting resistance to the commonly used antimicrobials. Nalidixic acid discs should be used to detect quinolone resistance.

- (i) Suspend colonies from a fresh plated culture in sterile distilled water. Adjust the density of the suspension to that of a 0.5 McFarland standard.
- (ii) Use a swab dipped in the undiluted suspension to inoculate evenly the entire surface of Iso-Sensitest agar supplemented with 5% horse blood.

- (iii) Incubate for 18-24 hours at 42°C in microaerophilic conditions. *Campylobacter fetus*, which is primarily associated with extraintestinal infections, does not grow well at 42°C and should be incubated at 35-37°C.
- (iv) Measure the zone diameters. Tentative zone breakpoints are given in Table III.

Brucella spp.

Brucella spp. are Hazard Group 3 pathogens and all work must be done in containment level 3 accommodation. The antimicrobial agents most commonly used for treatment are doxycycline, rifampicin, ciprofloxacin, tetracycline and streptomycin and, from the limited information available, there is little or not resistance to these drugs. *Brucella* spp. are uncommon isolates and interpretative standards are not available. Since *Brucella* spp. are highly infectious, the Working Party recommends that no susceptibility test is done in clinical laboratories.

Legionella spp.

Legionella spp. are slow growing and have particular growth requirements. Disc diffusion methods for susceptibility tests are unsuitable. Susceptibility should be determined by agar dilution MICs on buffered yeast extract agar with 5% water-lysed horse blood.⁵ The antimicrobial agents commonly used for treatment are macrolides, rifampicin and fluoroquinolones. Validated MIC breakpoints are not established for *Legionella* spp. If results for test isolates are within the range of the normal wild-type distribution, listed in Table IV,⁵⁻⁷ clinical susceptibility may be assumed.

Stenotrophomonas maltophilia

S. maltophilia is most often associated with colonization, but is an occasional cause of infection. There is no data, at present, to support a relationship between laboratory susceptibility testing and clinical outcome with *S. maltophilia* infection.

Susceptibility testing of *S. maltophilia* is particularly difficult and MICs and zones for the species are affected by both temperature and medium. Many isolates grow better at 30°C and some isolates grow poorly, or not at all, at 37°C. The activities of aminoglycosides, and polymyxins against the species are particularly vulnerable to temperature variation and isolates often appear falsely susceptible at 37°C. Isolates should nevertheless be reported resistant to these drugs, and to carbapenems irrespective of zone diameters.

The drug of choice for treatment is co-trimoxazole and testing of this drug on Iso-Sensitest agar is satisfactory. Tentative zone diameter breakpoints for 25 µg content discs are ≤19 mm for resistant and ≥20 mm for susceptible. Results with trimethoprim alone are not predictive of susceptibility or resistance to co-trimoxazole and results should not be extrapolated. There is some clinical evidence that the addition of other agents such as quinolones (moxifloxacin is the most active) to co-trimoxazole may be therapeutically advantageous. Testing of quinolones by disc diffusion is difficult, with the results affected by the temperature, and zone breakpoints have not been ascertained. Nevertheless, combination therapy may be indicated irrespective of the laboratory results.

In patients where co-trimoxazole is not a suitable agent for treatment (owing to resistance of the isolate or, more commonly sulphonamide intolerance of the patient), other antimicrobials that are known to have limited activity are the β-lactams ticarcillin/clavulanate, aztreonam plus co-amoxiclav, and ceftazidime in combination with other agents. These β-lactams often appear active against isolates of *S. maltophilia* on Iso-Sensitest agar but will appear less active if tested on Mueller-Hinton agar. There is no good evidence available to relate this anomaly to in-vivo activity. This advice was compiled with the help of Dr R Howe Consultant Microbiologist at Southmead Hospital in Bristol.

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Table I. Tentative zone diameter breakpoints for rapidly growing anaerobic organisms

Antibiotic	MIC breakpoint (mg/L)		Disc content (μ g)	Interpretation of zone diameter (mm)	
	R	S		R	S
Penicillin	≥ 2	≤ 1	2	≤ 17	≥ 18
Clindamycin	≥ 4	≤ 2	2	≤ 17	≥ 18
Metronidazole	≥ 16	≤ 8	5	≤ 17	≥ 18

Table II. Tentative MIC breakpoints for *H. pylori*

Antibiotic	MIC breakpoint (mg/L)	
	R	S
Amoxycillin	≥ 2	≤ 1
Clarithromycin	≥ 2	≤ 1
Tetracycline	≥ 4	≤ 2
Metronidazole	≥ 8	≤ 4

Table III. Tentative zone diameter breakpoints for *Campylobacter* spp.

Antibiotic	MIC breakpoint (mg/L)		Disc content (μ g)	Interpretation of zone diameter (mm)	
	R	S		R	S
Erythromycin	≥ 2	≤ 1	5	≤ 19	≥ 20
Ciprofloxacin	≥ 4	≤ 2	1	≤ 17	≥ 18
Nalidixic acid			30	≤ 15	≥ 16

Table IV. MIC ranges for normal wild-type *Legionella* spp.

Antibiotic	Range of MICs (mg/L)
Erythromycin	0.06-0.5
Clarithromycin	0.004-0.06
Rifampicin	0.004-0.06
Ciprofloxacin	0.016-0.06