Chapter 1-3. Anaerobic infections (general): drug susceptibility tests

Performing drug susceptibility tests

Drug susceptibility tests for anaerobes accompany technical difficulties, as compared to similar tests for aerobic bacteria in general, for the following reasons: (1) special manipulation to avoid contamination by ambient oxygen is needed; (2) anaerobic culture is needed; (3) the growth rate varies greatly among different types of anaerobes; and (4) many anaerobes have high nutritional requirements. Anaerobic infection more frequently involved in multiple bacterial infections than the single bacterial form of infection. For example, 3–5 species of anaerobes are isolated on average from each case of suppurative anaerobic infection encountered in the field of surgery. At hospital laboratories, a prevailing view is that unlike the bacterial strains isolated from blood or cerebrospinal fluid, bacterial strains isolated from suppurative lesions do not always need to be checked as to drug susceptibility in a routine manner. Performing drug susceptibility tests on all such strains is also unfavorable from the standpoint of medical economics. However, it is not uncommon for resistance to various antibacterial agents to be exhibited by anaerobes isolated from clinical samples. Furthermore, the trend in resistance of anaerobes to drugs can change, like the trend in the resistance of aerobes. Still more, it is not rare for serious outcomes to manifest if antimicrobial chemotherapy is administered, ignoring the possible presence of anaerobes, in patients with major infections known to involve anaerobes.

Under the circumstances described above, it is thought to be desirable that chemotherapy for suppurative infections, which account for a high percentage of anaerobic infections, be based on empirical treatment with antibiograms taken into account, and that the therapy be modified on the basis of the results of drug susceptibility tests performed as needed. Clearly, the importance of antibiograms during routine clinical practice is higher when performing chemotherapy for anaerobes than for aerobes because the former does not involve routine drug susceptibility testing. Because antibiograms can vary among countries, districts or hospitals, it is essential that susceptibility tests be performed periodically on major bacteria within a country, a district or individual hospital to an extent practically feasible, to yield information about susceptibility patterns in a given area. When chemotherapy is performed on the basis of reported antibiograms, it must be borne in mind that there may be some differences in susceptibility patterns or the percentage of resistant strains among different districts or facilities.

Table 1 lists cases in which susceptibility testing should be performed on the isolated anaerobes. Table 2 shows major types of bacteria which require susceptibility tests.

Methods of drug susceptibility tests

Methods available for drug susceptibility evaluation include the agar dilution, broth microdilution, disc diffusion and cup methods. CLSI (Clinical and Laboratory Standards Institutes, formerly called NCCLS) [1] recommends the agar dilution and broth microdilution methods for assessment of the susceptibility of anaerobes to drugs. The E test is also considered to be useful as an alternative method. These methods will be outlined below. See relevant references for details of the standard method recommended by the Japanese Society of Chemotherapy, the CLSI method and E test.

The disc diffusion method, which is recommended for aerobes, is not recommended for assessment of the
susceptibility of anaerobes. This simple method is useful when obtaining reference data, to be used within a given hospital, on bacterial strains which grow well in about 48 h in media suitable for their growth. From academic points of view, however, the data yielded by this test are not formally acceptable as susceptibility data.

Agar dilution method

Agar media, containing drugs in serially twofold diluted concentrations, are prepared in different dishes. A certain amount of the test bacterial strain is then inoculated onto each dish. Inhibition of bacterial growth on the agar medium is then observed in steps, to determine the minimum concentration allowing the test strain to grow.

This method of susceptibility assessment has undergone repeated assessments. At present, it is recommended as the most reliable means of measuring the MIC of drugs against anaerobes in general. The MIC determined by the agar dilution method is used as reference information when assessing the validity of other methods of MIC measurement. This method is used for susceptibility surveillance and evaluation of new drugs. With this method, the susceptibility of multiple bacterial strains can be assessed at a single measurement. However, since it requires diverse supplies (reagents, devices, etc.) and considerable labor and time in preparation for the test, this method is not suitable for susceptibility testing carried out routinely in laboratories.

The standard method formerly recommended by the Japanese Society of Chemotherapy [2] conventionally used modified GAM agar media, etc., for susceptibility assessment and specified an amount of bacteria to be inoculated and the drug concentration unique to Japan (different from those used in foreign countries) [2]. Recently, however, this method was revised to update the specifications given in the CLSI standard. The revised standard method (“Standard Agar Dilution Method of the Japanese Society of Chemotherapy (Revised)”, reported by the Committee for Japanese Standards for Antimicrobial Susceptibility testing for Bacteria at the 52nd General Meeting of the Japanese Society of Chemotherapy) is separately described later.

Broth microdilution method

Liquid media, containing drugs in serially twofold diluted concentrations, are prepared on the wells of a microtiter plate. A certain amount of the test bacterial strain is inoculated onto each well. Inhibition of bacterial growth in each liquid medium is then observed in steps, to determine the minimum concentration allowing the test strain to grow.

With this method, susceptibilities of the test strain to multiple drugs can be assessed using a single microtiter plate. Manipulations required are simpler with this method than with agar dilution method. This method is suitable for routine susceptibility testing, i.e., for assessment of susceptibility of individual samples brought to the laboratory in daily practice.

The standard broth microdilution method, recommended by the Japanese Society of Chemotherapy [3], is the frozen plate technique by which a microtiter plate carrying liquid media containing drugs is stored frozen and then subjected to thawing and preliminary reduction before inoculation with 1/100 its volume of test bacterial suspension. With
Table 3  Drug susceptibility test “Standard Agar Dilution Method Recommended by the Japanese Society of Chemotherapy (revised)”

[Target bacteria]
Anaerobes (anaerobes growing well in media designated for use in susceptibility testing)

[Media for susceptibility testing]
Brucella agar medium supplemented with hemin (5 μg/mL), menadione (1 μg/mL) and lysed sheep blood (5%)

[Preparation of bacterial suspension]

- **Growth broth suspension method**
  A thioglycolate medium supplemented with hemin and menadione (containing no indicator agent) is inoculated with a sufficient number of colonies. Then, incubation is carried out in an incubator until the medium becomes appropriately opaque (for 6–24 h)

- **Direct colony suspension method**
  Colonies, incubated for 24–48 h in an appropriate agar medium for anaerobe culture, are directly suspended in a liquid medium for preparing bacterial suspensions. This step should be completed within 30 min after the anaerobic environment is suspended

[Preparation procedure]
With any of the growth broth suspension method and with the direct colony suspension method, a bacterial suspension with an optical density level equal to that of the 0.5 McFarland standard solution is prepared, as a rule [Optical density level needs to be adjusted depending on the type of bacterium. See supplementary provisions in “the Report by the Committee for Japanese Standards for Antimicrobial Susceptibility Testing for Bacteria, the Japanese Society of Chemotherapy (1992)”, vol. 41, p. 183, Journal of the Chemical Society of Japan]. Highly transparent liquid media (Brucella broth, Anaerobe broth, etc.) should be used as the liquid media for preparing bacterial suspensions. They need to be reduced or degassed (by boiling and subsequent rapid cooling) before use. Each bacterial suspension should be prepared immediately before inoculation

[Preparation of antibacterial agents]

- **Types of antibacterial drugs**
  Equivalent to those used for facultative bacteria

- **Drug dissolving method**
  The drug is prepared at the concentration of serial twofold dilution, including 1 μg/mL in the line of concentration

[Preparation of the agar plate]

- **Method**
  1. The medium for susceptibility testing is kept warmed at 48–50°C in advance
  2. Each antibacterial drug solution is combined with the medium for susceptibility testing at a ratio of 1:9
    (1) The agar thickness is 3–4 mm
    (2) Agar medium is left standing at room temperature and is thereby induced to solidify. The medium is used immediately after it has solidified

- **Storage**
  As a rule in Japan, the agar plate is used within the same day and is not stored

[Inoculation]
The bacterial suspension is inoculated onto the medium in a volume of 1–2 μL. The final density of bacteria inoculated should be $10^7$ cfu/spot. Because many anaerobes can endure exposure to ambient air if the exposure is brief, all manipulations related to inoculation can be done in open air. However, depending on the type or strain of bacterium, all manipulations need to be done within an anaerobic glove box

- **Precautions**
  1. At the beginning and end of inoculation of bacterial suspension, two drug-free control media (one for control of anaerobic culture and the other for control of aerobic culture) are also inoculated with the bacterial suspension to assess the growth of anaerobes and check for contamination by facultative bacteria
  2. Inoculation begins with a plate containing the lowest concentration of the drug and ends with the plate containing the highest concentration of the drug
  3. When the drug type is changed, a drug-free medium is additionally inoculated with the bacterial suspension to confirm the absence of any influence from the previous drug

[Culture]

- **Environments**
  An anaerobic glove box or one of the containers for anaerobic culture is used

- **Precautions**
  According to the CLSI method, the carbon dioxide concentration should be 4–7% (corresponding to the concentration for the gas pack method). Bacterial growth is often stimulated as the carbon dioxide concentration rises, but the concentration may not exceed approximately 10%. When reporting the results, the carbon dioxide concentration in the environment should be described
this technique, the susceptibility of many clinically isolated anaerobes can be assessed by adequate preliminary reduction of the media used. Another broth microdilution method available is the dry plate technique by which the freeze-dried drug is placed in advance in each well of the microtiter plate and a certain volume of test bacterial suspension prepared with the susceptibility assay medium added to the well before incubation. This technique, which does not require thawing and preliminary reduction, is more convenient than the frozen plate technique and is often used in clinical laboratories. However, the dry plate technique is not identical to the frozen plate technique recommended as the standard method by the Japanese Society of Chemotherapy. With the dry plate technique, the entire inoculated bacterial suspension volume concurrently serves as the medium for susceptibility assessment. For this reason, when the liquid medium adjusted to contain a certain amount of test bacterium is poured into the plate while exposed to ambient air, the status of preliminary reduction of the medium differs from that in the medium used for the frozen plate technique.

When a liquid medium free of blood and other supplements is used for the dry plate technique, there are large numbers of clinically significant bacteria which do not show growth in this medium, such as black-pigmented bacteria (Prevotella species, Porphyromonas species), Fusobacterium species, anaerobic cocci, and so on, unlike the growth of these types of bacteria in medium used for the frozen plate technique. If the recently marketed medium (a Brucella broth supplemented with lysed horse blood, hemin and vitamin K1), recommended by CLSI (NCCLS M11-A6) as a medium for the broth microdilution method, is used, even the dry plate technique allows assessment of the susceptibility of many anaerobic bacteria including those mentioned above in a generally satisfactory manner. The dry plate technique is extensively applicable to routine susceptibility assessment as a substitute for the frozen plate technique. CLSI (NCCLS M11-A6) does not refer to the necessity of routine preliminary reduction for each testing occasion when a frozen plate prepared at a given facility is used or when a commercial frozen plate or dry plate is used. However, according to the CLSI recommendation, the use of this technique is limited to the B. fragilis group on the grounds that the validity of this technique has not yet been adequately endorsed for other bacteria. When broth microdilution is performed using either a plate prepared at a given facility or a commercial plate, in any technique (frozen plate or dry plate) employed, it is essential that the prescribed volume of the bacterial suspension is followed, that the final fluid volume is equal to 100 μL/well, and that each plate has wells for growth control.

E test

One side of the plastic strip, used for this method, is coated with an antibacterial drug in a concentration gradient. The concentrations used are labeled on the strip surface. A certain amount of the test bacterial suspension is inoculated homogenously onto the agar plate, and this strip is placed on the agar plate, followed by anaerobic culture. As a principle, the concentration at the point of crossing

Table 3 continued

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<thead>
<tr>
<th>Culture conditions</th>
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<tr>
<td>Anaerobic culture is performed for 42–48 h at 35–37°C. To confirm the absence of contamination by facultative bacteria, culture under carbon dioxide should be performed on one of the control media inoculated with the bacterial suspension before the start of anaerobic culture and another such medium after the start of anaerobic culture.</td>
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[Reading endpoint]

The concentration (endpoint) is determined macroscopically under adequate illumination, referring to the criteria shown below. *Reference

1. Bacterial growth is markedly suppressed and no bacterial growth is visible at a given concentration
2. Bacterial growth gradually decreases, and only slight growth or a general tendency for haze formation (traces of inoculated bacterial suspension visible as groups of very small spots) is visible at a given concentration
3. Bacterial growth is markedly suppressed, but many small colonies persist at a given concentration
4. Bacterial growth is markedly suppressed, but several colonies of normal size persist at a given concentration
5. Bacterial growth is markedly suppressed, but haze persists at a given concentration
6. Bacterial growth gradually decreases, and a small number of large colonies are visible at a given concentration

[Quality control]

1. Bacteroides fragilis ATCC25285
2. Bacteroides thetaiotaomicron ATCC29741
3. Eggerthella lenta ATCC43055

Reported by the Committee for Japanese Standards for Antimicrobial Susceptibility testing for Bacteria at the 52nd General Meeting of the Japanese Society of Chemotherapy.
between the margin of the growth inhibited zone and the strip indicates the MIC. This strip is a commercially developed product based on a novel idea. Although CSLI does not officially refer to this product, it has been accepted as a useful means of susceptibility evaluation. Because this method uses a medium allowing good growth of the test strain, it is applicable to assessment of the susceptibility of a wide range of bacteria, including black-pigmented bacteria. It is also reported to be well correlated with agar dilution method. However, it has been reported that the validity of this strip is questionable when used on some types of bacteria and drugs. It is advisable for users of this strip to obtain information about the types of bacteria or drugs which require particular care when using this product from the manufacturer or distributor at the time of purchase. This strip does not require complex manipulations. It can be used easily even for samples containing a small number of bacterial strains. Many types of bacteria can be assessed with this strip. Because of these features, this strip is convenient for routine susceptibility testing, but it is not inexpensive.

Quality control

Even when the laboratory staff performs susceptibility assessment in accordance with a given set of procedures, the reliability of the test cannot always be assured if there are problems with the medium, drug, performance of the plate or manipulation employed. To avoid this type of problem, it has been recommended that on each occasion the susceptibility test should include a bacterial strain for quality control. Bacterial strains recommended for use in quality control are *B. fragilis* ATCC25285, *B. thetaiotaomicron* ATCC29741 and *Eggerthella lenta* ATCC43055. According to the standard susceptibility assessment method recommended by the Japanese Society of Chemotherapy, *B. fragilis* GAI 5524 and *B. thetaiotaomicron* GAI 5628 can also be used as bacterial strains for quality control. It has been recommended that 2 or more of the 3 control strains be used when the agar dilution method is employed and to use at least one control strain when the broth microdilution method is employed. The MIC of several indicator drugs against these strains have been presented for both the agar dilution and the broth microdilution method. The readings of measurements for these control strains must be within the acceptable ranges presented. See the standard method recommended by the Japanese Society of Chemotherapy [2, 3] and CLSI [1] for details. When susceptibility is assessed using commercial kits, quality control should be performed in accordance with the procedure described in the manufacturer’s instruction (Table 3).

References