

e14 Laboratory Diagnosis of Infectious Diseases

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The laboratory diagnosis of infection requires the demonstration—either direct or indirect—of viral, bacterial, fungal, or parasitic agents in tissues, fluids, or excreta of the host. Clinical microbiology laboratories are responsible for processing these specimens and also for determining the antibiotic susceptibility of bacterial and fungal pathogens. Traditionally, detection of pathogenic agents has relied largely on either the microscopic visualization of pathogens in clinical material or the growth of microorganisms in the laboratory. Identification is generally based on phenotypic characteristics, such as fermentation profiles for bacteria, cytopathic effects in tissue culture for viral agents, and microscopic morphology for fungi and parasites. These techniques are reliable but are often time-consuming. Increasingly, the use of nucleic acid probes is becoming a standard method for detection, quantitation, and/or identification in the clinical microbiology laboratory, gradually replacing phenotypic characterization and microscopic visualization methods.

DETECTION METHODS

Reappraisal of the methods employed in the clinical microbiology laboratory has led to the development of strategies for detection of pathogenic agents through nonvisual biologic signal detection systems. Much of this methodology is based on the use of either electronic detection systems involving relatively inexpensive but sophisticated computers or nucleic acid probes directed at specific DNA or RNA targets. This chapter discusses both the methods that are currently available and those that are being developed.

BIOLOGIC SIGNALS

A *biologic signal* is a material that can be reproducibly differentiated from other substances present in the same physical environment. Key issues in the use of a biologic (or electronic) signal are distinguishing it from background noise and translating it into meaningful information. Examples of biologic signals applicable to clinical microbiology include structural components of bacteria, fungi, and viruses; specific antigens; metabolic end products; unique DNA or RNA base sequences; enzymes; toxins or other proteins; and surface polysaccharides.

DETECTION SYSTEMS

A detector is used to sense a signal and to discriminate between the signal and background noise. Detection systems range from the trained eyes of a technologist assessing morphologic variations to sensitive electronic instruments, such as gas-liquid chromatographs coupled to computer systems for signal analysis. The sensitivity with which signals can be detected varies widely. It is essential to use a detection system that discerns small amounts of signal even when biologic background noise is present—i.e., that is both sensitive and specific. Common detection systems include immunofluorescence; chemiluminescence for DNA/RNA probes; flame ionization detection of short- or long-chain fatty acids; and detection of substrate utilization or end-product formation as color changes, of enzyme activity as a change in light absorbance, of turbidity changes as a measure of growth, of cytopathic effects in cell lines, and of particle agglutination as a measure of antigen presence.

AMPLIFICATION

Amplification enhances the sensitivity with which weak signals can be detected. The most common microbiologic amplification technique is growth of a single bacterium into a discrete colony on an agar plate or into a suspension containing many identical organisms. The advantage of growth as an amplification method is that it requires only

an appropriate growth medium; the disadvantage is the amount of time required for amplification. More rapid specific amplification of biologic signals can be achieved with techniques such as polymerase chain reaction (PCR) or ligase chain reaction (LCR, for DNA/RNA), enzyme immunoassays (EIAs, for antigens and antibodies), electronic amplification (for gas-liquid chromatography assays), antibody capture methods (for concentration and/or separation), and selective filtration or centrifugation. Although a variety of methods are available for the amplification and detection of biologic signals in research, thorough testing is required before these methods are validated as diagnostic assays.

DIRECT DETECTION

MICROSCOPY

The field of microbiology has been defined largely by the development and use of the microscope. The examination of specimens by microscopic methods rapidly provides useful diagnostic information. Staining techniques permit organisms to be seen more clearly.

The simplest method for microscopic evaluation is the wet mount, which is used, for example, to examine cerebrospinal fluid (CSF) for the presence of *Cryptococcus neoformans*, with India ink as a background against which to visualize large-capsuled yeast cells. Wet mounts with dark-field illumination are also used to detect spirochetes from genital lesions and to reveal *Borrelia* or *Leptospira* in blood. Skin scrapings and hair samples can be examined with use of either 10% KOH wet-mount preparations or the calcofluor white method and ultraviolet illumination to detect fungal elements as fluorescing structures. Staining of wet mounts—e.g., with lactophenol cotton blue stain for fungal elements—is often used for morphologic identification. These techniques enhance signal detection and decrease the background, making it easier to identify specific fungal structures.

STAINING

Gram's Stain Without staining, bacteria are difficult to see at the magnifications (400× to 1000×) used for their detection. Although simple one-step stains can be used, differential stains are more common. Gram's stain differentiates between organisms with thick peptidoglycan cell walls (gram-positive) and those with thin peptidoglycan cell walls and outer membranes that can be dissolved with alcohol or acetone (gram-negative). Cellular morphology and Gram's stain characteristics can often be used to categorize stained organisms into groups such as streptococci, staphylococci, and clostridia (Fig. e14-1).

Gram's stain is particularly useful for examining sputum for polymorphonuclear leukocytes (PMNs) and bacteria. Sputum specimens from immunocompetent patients with ≥ 25 PMNs and < 10 epithelial cells per low-power field often provide clinically useful information. However, the presence in "sputum" samples of > 10 epithelial cells per low-power field and of multiple bacterial types suggests contamination with oral microflora. Despite the difficulty of discriminating between normal microflora and pathogens, Gram's stain may prove useful for specimens from areas with a large resident microflora if a useful biologic marker (signal) is available. Gram's staining of vaginal swab specimens can be used to detect epithelial cells covered with gram-positive bacteria in the absence of lactobacilli and the presence of gram-negative rods—a scenario regarded as a sign of bacterial vaginosis. Similarly, examination of stained stool specimens for leukocytes is useful as a screening procedure before testing for *Clostridium difficile* toxin or other enteric pathogens.

The examination of CSF and joint, pleural, or peritoneal fluid with Gram's stain is useful for determining whether bacteria and/or PMNs are present. The sensitivity is such that $> 10^4$ bacteria per milliliter should be detected. Centrifugation is often performed before staining to concentrate specimens thought to contain low numbers of organisms. The pellet is examined after staining. This simple method is particularly useful for examination of CSF for bacteria and white blood cells or of sputum for acid-fast bacilli (AFB).

	GRx only	Oxidase +	Oxidase –	Fastidious	Anaerobic	Curved
Rod		<i>Pseudomonas</i> <i>Aeromonas</i> <i>Pasteurella</i> Others	Enterobacteriaceae Others	<i>Haemophilus</i> <i>Legionella</i> <i>Bordetella</i> <i>Brucella</i> <i>Francisella</i> Others	<i>Bacteroides</i> <i>Prevotella</i> <i>Fusobacterium</i> Others	<i>Vibrio</i> <i>Campylobacter</i>
Coccus	<i>Neisseria</i> <i>Branhamella</i>				<i>Veillonella</i> <i>Acidaminococcus</i> <i>Megasphaera</i>	

Gram-Positive Organisms

	Branching	Spores	Acid-Fast	Catalase +	Catalase –
Rod	<i>Nocardia</i> <i>Actinomyces</i> <i>Bifidobacterium</i>	<i>Clostridium</i> <i>Bacillus</i>	<i>Mycobacterium</i>	<i>Corynebacterium</i> <i>Listeria</i> Others	<i>Lactobacillus</i> Others
Coccus				<i>Staphylococcus</i> <i>Micrococcus</i> Others	<i>Streptococcus</i>

FIGURE e14-1 Interpretation of Gram's stain.

Acid-Fast Stain The acid-fast stain identifies organisms that retain carbol fuchsin dye after acid/organic solvent disruption (e.g., *Mycobacterium* spp.). Modifications of this procedure allow the differentiation of *Actinomyces* from *Nocardia* or other weakly (or partially) acid-fast organisms. The acid-fast stain is applied to sputum, other fluids, and tissue samples when AFB (e.g., *Mycobacterium* spp.) are suspected. The identification of the pink/red AFB against the blue background of the counterstain requires a trained eye, since few AFB may be detected in an entire smear, even when the specimen has been concentrated by centrifugation. An alternative method is the auramine-rhodamine combination fluorescent dye technique.

Fluorochrome Stains Fluorochrome stains, such as acridine orange, are used to identify white blood cells, yeasts, and bacteria in body fluids. Other specialized stains, such as Dappe's stain, may be used for the detection of mycoplasmas in cell cultures. Capsular, flagellar, and spore stains are used for identification or demonstration of characteristic structures.

Immunofluorescent Stains The *direct* immunofluorescent antibody technique uses antibody coupled to a fluorescing compound, such as fluorescein, and directed at a specific antigenic target to visualize organisms or subcellular structures. When samples are examined under appropriate conditions, the fluorescing compound absorbs ultraviolet light and reemits light at a higher wavelength visible to the human eye. In the *indirect* immunofluorescent antibody technique, an unlabeled (target) antibody binds a specific antigen. The specimen is then stained with fluorescein-labeled polyclonal antibody directed at the target antibody. Because each unlabeled target antibody attached to the appropriate antigen has multiple sites for attachment of the second antibody, the visual signal can be intensified (i.e., amplified). This form of staining is called *indirect* because a two-antibody system is used to generate the signal for detection of the antigen. Both direct and indirect methods detect viral antigens (e.g., cytomegalovirus, herpes simplex virus, and respiratory viruses) within cultured cells or clinical specimens as well as many difficult-to-grow bacterial agents (e.g., *Legionella pneumophila*) directly in clinical specimens.

MACROSCOPIC ANTIGEN DETECTION

Latex agglutination assays and EIAs are rapid and inexpensive methods for identifying organisms, extracellular toxins, and viral agents by means of protein and polysaccharide antigens. Such assays may be performed directly on clinical samples or after growth of organisms on agar plates

or in viral cell cultures. The biologic signal in each case is the antigen to be detected. Monoclonal or polyclonal antibodies coupled to a reporter (such as latex particles or an enzyme) are used for detection of antibody-antigen binding reactions.

Techniques such as direct agglutination of bacterial cells with specific antibody are simple but relatively insensitive, while latex agglutination and EIAs are more sensitive. Some cell-associated antigens, such as capsular polysaccharides and lipopolysaccharides, can be detected by agglutination of a suspension of bacterial cells when antibody is added; this method is useful for typing of the somatic antigens of *Shigella* and *Salmonella*. In systems such as EIAs, which employ antibodies coupled to an enzyme, an antigen-antibody reaction results in the conversion of a colorless substrate to a colored product. Because the coupling of an enzyme to the antibody can amplify a weak biologic signal, the sensitivity of such assays is often high. In each instance, the basis for antigen detection is antigen-antibody binding, with the detection system changed to accommodate the biologic signal. Most such assays provide information as to whether antigen is present but do not quantify the antigen. EIAs are also useful for detecting bacterial toxins—e.g., *C. difficile* toxins A and B in stool.

Rapid and simple tests for antigens of group A *Streptococcus*, influenza virus, and respiratory syncytial virus can be used in the clinic setting, without a specialized diagnostic laboratory. Such tests are usually reasonably specific but may have only modest sensitivity.

DETECTION OF PATHOGENIC AGENTS BY CULTURE

SPECIMEN COLLECTION AND TRANSPORT

To culture bacterial, mycotic, or viral pathogens, an appropriate sample must be placed into the proper medium for growth (amplification). The success of efforts to identify a specific pathogen often depends on the collection and transport process coupled to a laboratory-processing algorithm suitable for the specific sample/agent. In some instances, it is better for specimens to be plated at the time of collection rather than first being transported to the laboratory (e.g., urethral swabs being cultured for *Neisseria gonorrhoeae* or sputum specimens for pneumococci). In general, the more rapidly a specimen is plated onto appropriate media, the better the chance for isolating bacterial pathogens. Deep tissue or fluid (pus) samples are more likely to give useful culture results than are superficial swab specimens. **Table e14-1** lists procedures for collection and transport of common specimens. Because there are many pathogen-specific paradigms for these procedures, it is important to seek advice from the microbiology laboratory when in doubt about a particular situation.

ISOLATION OF BACTERIAL PATHOGENS

Isolation of suspect pathogens from clinical material relies on the use of artificial media that support bacterial growth *in vitro*. Such media are composed of agar, which is not metabolized by bacteria; nutrients to support the growth of the species of interest; and sometimes substances to inhibit the growth of other bacteria. Broth is employed for growth (amplification) of organisms from specimens with few bacteria, such as peritoneal dialysis fluid, CSF, or samples in which anaerobes or other fastidious organisms may be present. The general use of liquid medium for all specimens is not worthwhile.

TABLE e14-1 INSTRUCTIONS FOR COLLECTION AND TRANSPORT OF SPECIMENS FOR CULTURE

Note: It is absolutely essential that the microbiology laboratory be informed of the site of origin of the sample to be cultured and of the infections that are suspected. This information determines the selection of culture media and the length of culture time.

Type of Culture (Synonyms)	Specimen	Minimum Volume	Container	Other Considerations
Blood				
Blood, routine (blood culture for aerobes, anaerobes, and yeasts)	Whole blood	10 mL in each of 2 bottles for adults and children; 5 mL, if possible, in aerobic bottles for infants; less for neonates	See below. ^a	See below. ^b
Blood for fungi/ <i>Mycobacterium</i> spp.	Whole blood	10 mL in each of 2 bottles, as for routine blood cultures, or in Isolator tube requested from laboratory	Same as for routine blood culture	Specify "hold for extended incubation," since fungal agents may require ≥ 4 weeks to grow.
Blood, Isolator (lysis centrifugation)	Whole blood	10 mL	Isolator tubes	Use mainly for isolation of fungi, <i>Mycobacterium</i> , or other fastidious aerobes and for elimination of antibiotics from cultured blood in which organisms are concentrated by centrifugation.
Respiratory Tract				
Nose	Swab from nares	1 swab	Sterile culturette or similar transport system containing holding medium	Swabs made of calcium alginate may be used.
Throat	Swab of posterior pharynx, ulcerations, or areas of suspected purulence	1 swab	Sterile culturette or similar swab specimen collection system containing holding medium	See below. ^c
Sputum	Fresh sputum (not saliva)	2 mL	Commercially available sputum collection system or similar sterile container with screw cap	<i>Cause for rejection:</i> Care must be taken to ensure that the specimen is sputum and not saliva. Examination of Gram's stain, with number of epithelial cells and PMNs noted, can be an important part of the evaluation process. Induced sputum specimens should not be rejected.
Bronchial aspirates	Transtracheal aspirate, bronchoscopy specimen, or bronchial aspirate	1 mL of aspirate or brush in transport medium	Sterile aspirate or bronchoscopy tube, bronchoscopy brush in a separate sterile container	Special precautions may be required, depending on diagnostic considerations (e.g., <i>Pneumocystis</i>).
Stool				
Stool for routine culture; stool for <i>Salmonella</i> , <i>Shigella</i> , and <i>Campylobacter</i>	Rectal swab or (preferably) fresh, randomly collected stool	1 g of stool or 2 rectal swabs	Plastic-coated cardboard cup or plastic cup with tight-fitting lid. Other leak-proof containers are also acceptable.	If <i>Vibrio</i> spp. are suspected, the laboratory must be notified, and appropriate collection/transport methods should be used.
Stool for <i>Yersinia</i> , <i>E. coli</i> O157	Fresh, randomly collected stool	1 g	Plastic-coated cardboard cup or plastic cup with tight-fitting lid	<i>Limitations:</i> Procedure requires enrichment techniques.
Stool for <i>Aeromonas</i> and <i>Plesiomonas</i>	Fresh, randomly collected stool	1 g	Plastic-coated cardboard cup or plastic cup with tight-fitting lid	<i>Limitations:</i> Stool should not be cultured for these organisms unless also cultured for other enteric pathogens.
Urogenital Tract				
Urine	Clean-voided urine specimen or urine collected by catheter	0.5 mL	Sterile, leak-proof container with screw cap or special urine transfer tube	See below. ^d
Urogenital secretions	Vaginal or urethral secretions, cervical swabs, uterine fluid, prostatic fluid, etc.	1 swab or 0.5 mL of fluid	Vaginal and rectal swabs transported in Amies transport medium or similar holding medium for group B <i>Streptococcus</i> ; direct inoculation preferred for <i>Neisseria gonorrhoeae</i>	Vaginal swab samples for "routine culture" should be discouraged whenever possible unless a particular pathogen is suspected. For detection of multiple organisms (e.g., group B <i>Streptococcus</i> , <i>Trichomonas</i> , <i>Chlamydia</i> , or <i>Candida</i> spp.), 1 swab per test should be obtained.
Body Fluids, Aspirates, and Tissues				
Cerebrospinal fluid (lumbar puncture)	Spinal fluid	1 mL for routine cultures; ≥ 5 mL for <i>Mycobacterium</i>	Sterile tube with tight-fitting cap	Do not refrigerate; transfer to laboratory as soon as possible.
Body fluids	Aseptically aspirated body fluids	1 mL for routine cultures	Sterile tube with tight-fitting cap. Specimen may be left in syringe used for collection if the syringe is capped before transport.	For some body fluids (e.g., peritoneal lavage samples), increased volumes are helpful for isolation of small numbers of bacteria.

(continued)

Type of Culture (Synonyms)	Specimen	Minimum Volume	Container	Other Considerations
Biopsy and aspirated materials	Tissue removed at surgery, bone, anticoagulated bone marrow, biopsy samples, or other specimens from normally sterile areas	1 mL of fluid or a 1-g piece of tissue	Sterile "culturette"-type swab or similar transport system containing holding medium. Sterile bottle or jar should be used for tissue specimens.	Accurate identification of specimen and source is critical. Enough tissue should be collected for both microbiologic and histopathologic evaluations.
Wounds	Purulent material or abscess contents obtained from wound or abscess without contamination by normal microflora	2 swabs or 0.5 mL of aspirated pus	Culturette swab or similar transport system or sterile tube with tight-fitting screw cap. For simultaneous anaerobic cultures, send specimen in anaerobic transport device or closed syringe.	<i>Collection:</i> Abscess contents or other fluids should be collected in a syringe (rather than with a swab) when possible to provide an adequate sample volume and an anaerobic environment.
Special Recommendations				
Fungi	Specimen types listed above may be used. When urine or sputum is cultured for fungi, a first morning specimen is usually preferred.	1 mL or as specified above for individual listing of specimens. Large volumes may be useful for urinary fungi.	Sterile, leak-proof container with tight-fitting cap	<i>Collection:</i> Specimen should be transported to microbiology laboratory within 1 h of collection. Contamination with normal flora from skin, rectum, vaginal tract, or other body surfaces should be avoided.
<i>Mycobacterium</i> (acid-fast bacilli)	Sputum, tissue, urine, body fluids	10 mL of fluid or small piece of tissue. Swabs should not be used.	Sterile container with tight-fitting cap	Detection of <i>Mycobacterium</i> spp. is improved by use of concentration techniques. Smears and cultures of pleural, peritoneal, and pericardial fluids often have low yields. Multiple cultures from the same patient are encouraged. Culturing in liquid media shortens the time to detection.
<i>Legionella</i>	Pleural fluid, lung biopsy, bronchoalveolar lavage fluid, bronchial/transbronchial biopsy. Rapid transport to laboratory is critical.	1 mL of fluid; any size tissue sample, although a 0.5-g sample should be obtained when possible.	—	—
Anaerobic organisms	Aspirated specimens from abscesses or body fluids	1 mL of aspirated fluid, 1 g of tissue, or 2 swabs	An appropriate anaerobic transport device is required. ^e	Specimens cultured for obligate anaerobes should be cultured for facultative bacteria as well. Fluid or tissue is preferred to swabs.
Viruses ^f	Respiratory secretions, wash aspirates from respiratory tract, nasal swabs, blood samples (including buffy coats), vaginal and rectal swabs, swab specimens from suspicious skin lesions, stool samples (in some cases)	1 mL of fluid, 1 swab, or 1 g of stool in each appropriate transport medium	Fluid or stool samples in sterile containers or swab samples in viral culturette devices (kept on ice but not frozen) are generally suitable. Plasma samples and buffy coats in sterile collection tubes should be kept at 4–8°C. If specimens are to be shipped or kept for a long time, freezing at –80°C is usually adequate.	Most samples for culture are transported in holding medium containing antibiotics to prevent bacterial overgrowth and viral inactivation. Many specimens should be kept cool but not frozen, provided they are transported promptly to the laboratory. Procedures and transport media vary with the agent to be cultured and the duration of transport.

^aFor samples from adults, two bottles (smaller for pediatric samples) should be used: one with dextrose phosphate, tryptic soy, or another appropriate broth and the other with thioglycollate or another broth containing reducing agents appropriate for isolation of obligate anaerobes. For children, from whom only limited volumes of blood can be obtained, only an aerobic culture should be done unless there is specific concern about anaerobic sepsis (e.g., with abdominal infections). For special situations (e.g., suspected fungal infection, culture-negative endocarditis, or mycobacteremia), different blood collection systems may be used (Isolator systems; see table).

^b*Collection:* An appropriate disinfecting technique should be used on both the bottle septum and the patient. Do not allow air bubbles to get into anaerobic broth bottles. *Special considerations:* There is no more important clinical microbiology test than the detection of blood-borne pathogens. The rapid identification of bacterial and fungal agents is a major determinant of patients' survival. Bacteria may be present in blood either continuously (as in endocarditis, overwhelming sepsis, and the early stages of salmonellosis and brucellosis) or intermittently (as in most other bacterial infections, in which bacteria are shed into the blood on a sporadic basis). Most blood culture systems employ two separate bottles containing broth medium: one that is vented in the laboratory for the growth of facultative and aerobic organisms and a second that is maintained under anaerobic conditions. In cases of suspected continuous bacteremia/fungemia, two or three samples should be drawn before the start of therapy, with additional sets obtained if fastidious organisms are thought to be involved. For intermittent bacteremia, two or three samples should be obtained at least 1 h apart during the first 24 h.

^cNormal microflora includes alpha-hemolytic streptococci, saprophytic *Neisseria* spp., diphtheroids, and *Staphylococcus* spp. Aerobic culture of the throat ("routine") includes

screening for and identification of beta-hemolytic *Streptococcus* spp. and other potentially pathogenic organisms. Although considered components of the normal microflora, organisms such as *Staphylococcus aureus*, *Haemophilus influenzae*, and *Streptococcus pneumoniae* will be identified by most laboratories, if requested. When *Neisseria gonorrhoeae* or *Corynebacterium diphtheriae* is suspected, a special culture request is recommended.

^d(1) Clean-voided specimens, midvoid specimens, and Foley or indwelling catheter specimens that yield ≥50,000 organisms/mL and from which no more than three species are isolated should have organisms identified. Neither indwelling catheter tips nor urine from the bag of a catheterized patient should be cultured. (2) Straight-catheterized, bladder-tap, and similar urine specimens should undergo a complete workup (identification and susceptibility testing) for all potentially pathogenic organisms, regardless of colony count. (3) Certain clinical problems (e.g., acute dysuria in women) may warrant identification and susceptibility testing of isolates present at concentrations of <50,000 organisms/mL.

^eAspirated specimens in capped syringes or other transport devices designed to limit oxygen exposure are suitable for the cultivation of obligate anaerobes. A variety of commercially available transport devices may be used. Contamination of specimens with normal microflora from the skin, rectum, vaginal vault, or another body site should be avoided. Collection containers for aerobic culture (such as dry swabs) and inappropriate specimens (such as refrigerated samples; expectorated sputum; stool; gastric aspirates; and vaginal, throat, nose, and rectal swabs) should be rejected as unsuitable.

^fLaboratories generally use diverse methods to detect viral agents, and the specific requirements for each specimen should be checked before a sample is sent.

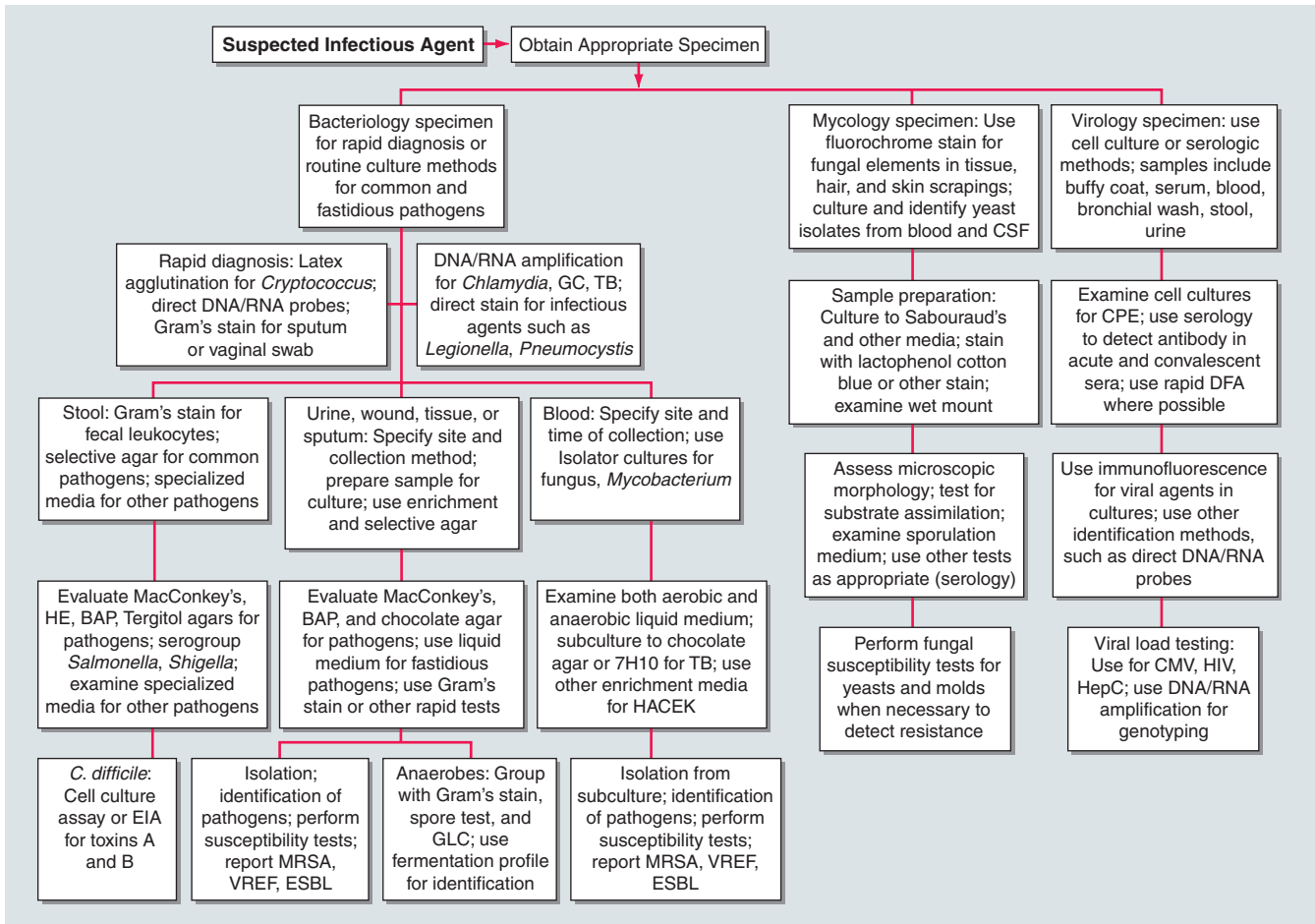


FIGURE e14-2 Common specimen-processing algorithms used in clinical microbiology laboratories.

Abbreviations: BAP, blood agar plate; CMV, cytomegalovirus; CPE, cytopathic effects; CSF, cerebrospinal fluid; DFA, direct fluorescent antibody; EIA, enzyme immunoassay; ESBL, extended-spectrum β -lactamase; GBS, group B *Streptococcus*; GC, *Neisseria gonorrhoeae*; GLC, gas-liquid chromatography; HACEK,

Haemophilus aphrophilus/parainfluenzae/paraphrophilus, *Actinobacillus actinomycetemcomitans*, *Cardiobacterium hominis*, *Eikenella corrodens*, and *Kingella kingae*; HE, Hektoen enteric medium; HepC, hepatitis C virus; HIV, human immunodeficiency virus; MRSA, methicillin-resistant *Staphylococcus aureus*; TB, *Mycobacterium tuberculosis*; VREF, vancomycin-resistant *Enterococcus faecium*.

Two basic strategies are used to isolate pathogenic bacteria. The first is to employ enriched media that support the growth of any bacteria that may be present in a sample such as blood or CSF, which contain no bacteria under normal conditions. Broths that allow the growth of small numbers of organisms may be subcultured to solid media when growth is detected. The second strategy is to use selective media to isolate (amplify) specific bacterial species from stool, genital tract secretions, or sputum—sites that contain many bacteria under normal conditions. Antimicrobial agents or other inhibitory substances are incorporated into the agar medium to inhibit growth of all but the bacteria of interest. After incubation, organisms that grow on such media are further characterized to determine whether they are pathogens. Selection for organisms that may be pathogens from the normal microflora shortens the time required for diagnosis (Fig. e14-2).

ISOLATION OF VIRAL AGENTS

(See also Chap. 170) Pathogenic viral agents often are sought by culture when the presence of serum antibody is not a criterion for active infection or when an increase in serum antibody may not be detected during infection. The biologic signal—virus—is amplified to a detectable level. Although a number of techniques are available, an essential element is a monolayer of cultured mammalian cells sensitive to infection with the suspected virus. These cells serve as the amplification system by allowing the proliferation of viral particles. Virus may be detected by direct observation of the cultured cells for cytopathic effects or by immunofluorescent detection of viral antigens following incuba-

tion. Conventional viral culture is useful for detection of rapidly propagated agents, such as herpes simplex virus. Viruses that grow more slowly (e.g., cytomegalovirus and varicella-zoster virus) can be detected quickly by shell-vial culture, in which the specimen is centrifuged on a monolayer of cells that is then incubated for 1–2 days and finally stained for viral antigens with fluorochrome-conjugated antibodies.

AUTOMATION OF MICROBIAL DETECTION IN BLOOD

The detection of microbial pathogens in blood is difficult because the number of organisms present in the sample is often low and the organisms' integrity and ability to replicate may be damaged by humoral defense mechanisms or antimicrobial agents. Over the years, systems that rely on the detection of gas (usually CO_2) produced by bacteria and yeasts in blood culture medium have allowed the automation of the detection procedure. The most common systems involve either (1) the measurement of gas pressure in the headspace to indicate bacterial gas production or consumption or (2) the use of reflectance optics, with a light-emitting diode and photodiode employed to detect a color change in a CO_2 -sensitive indicator built into the bottom of the culture bottle. These systems measure CO_2 concentration as indicative of microbial growth. Such methods are no more sensitive than the human eye in detecting a positive culture; however, because the bottles in an automated system are monitored more frequently, a positive culture is often detected more rapidly than by manual techniques, and important information, including the result of Gram's stain and pre-

e102 liminary susceptibility assays, can be obtained sooner. One advantage of automated blood culture systems is that the bottles are scanned continuously in a noninvasive monitoring procedure, and thus the likelihood of laboratory contamination is decreased.

Several factors affect the yield of blood culture from bacteremic patients. Increasing the volume of blood tested increases the chance of a positive culture. An increase from 10 to 20 mL of blood increases the proportion of positive cultures by ~30%; however, this effect is less pronounced in patients with bacterial endocarditis. Obtaining multiple cultures (up to three per 24-h period) also increases the chance of detecting a bacterial pathogen. Prolonged culture and blind subculture for detection of most fastidious bacteria (e.g., *Haemophilus*, *Actinobacillus*, *Cardiobacterium*, *Eikenella*, and *Kingella* spp.) are not needed with automated blood culture systems.

Automated systems have also been applied to the detection of microbial growth from specimens other than blood, such as peritoneal and other normally sterile fluids. *Mycobacterium* spp. can be detected in certain automated systems if appropriate liquid media are used for culture. Although automated blood culture systems are more sensitive than lysis-centrifugation methods (Isolator) for yeasts and most bacteria, lysis-centrifugation culture is recommended for filamentous fungi, *Histoplasma capsulatum*, and some fastidious bacteria (*Legionella* and *Bartonella*).

DETECTION OF PATHOGENIC AGENTS BY SEROLOGIC METHODS

Measurement of serum antibody provides an indirect marker for past or current infection with a specific viral agent or other pathogens, including *Brucella*, *Legionella*, *Rickettsia*, and *Helicobacter pylori*. The biologic signal is usually either IgM or IgG antibody directed at surface-exposed antigen. The detection systems include those used for bacterial antigens (agglutination reactions, immunofluorescence, and EIA) and unique systems such as hemolysis inhibition and complement fixation. Serologic methods generally fall into two categories: those that determine protective antibody levels and those that measure changing antibody titers during infection. Determination of an antibody response as a measure of current immunity is important in the case of viral agents for which there are vaccines, such as rubella virus or varicella-zoster virus; assays for this purpose normally use one or two dilutions of serum for a qualitative determination of protective antibody levels. Quantitative serologic assays to detect increases in antibody titers most often employ paired serum samples obtained at the onset of illness and 10–14 days later (i.e., acute- and convalescent-phase samples). Since the incubation period before symptoms are noted may be long enough for an antibody response to occur, the demonstration of acute-phase antibody alone is often insufficient to establish the diagnosis of active infection as opposed to past exposure. In such circumstances, IgM may be useful as a measure of an early, acute-phase antibody response. A fourfold increase in total antibody titer or in EIA activity between the acute- and convalescent-phase samples is also regarded as evidence for active infection.

For certain viral agents, such as Epstein-Barr virus, the antibodies produced may be directed at different antigens during different phases of the infection. For this reason, most laboratories test for antibody directed at both viral capsid antigens and antigens associated with recently infected host cells to determine the stage of infection.

IDENTIFICATION METHODS

Once bacteria are isolated, characteristics that are readily detectable after growth on agar media (colony size, color, hemolytic reactions, odor, microscopic appearance) may suggest a species, but definitive identification requires additional tests. Identification methods include classic biochemical phenotyping, which is still the most common approach, and more sophisticated methods such as gas chromatography and nucleic acid tests.

CLASSIC PHENOTYPING

Classic phenotypic identification of bacteria entails tests for protein or carbohydrate antigens, the production of specific enzymes, the ability

to metabolize specific substrates and carbon sources (such as carbohydrates), or the production of certain metabolites. Rapid versions of some of these tests are available, and many common organisms can be identified on the first day of growth. Other organisms, particularly gram-negative bacteria, require more extensive testing, either manual or automated.

Automated systems allow rapid phenotypic identification of bacterial pathogens. Most such systems are based on biotyping techniques, in which isolates are grown on multiple substrates and the reaction pattern is compared with known patterns for various bacterial species. This procedure is relatively fast, and commercially available systems include miniaturized fermentation, coding to simplify recording of results, and probability calculations for the most likely pathogens. If the biotyping approach is automated and the reading process is coupled to computer-based data analysis, rapidly growing organisms (such as Enterobacteriaceae) can be identified within hours of detection on agar plates.

Several systems use preformed enzymes for even speedier identification (within 2–3 h). Such systems do not rely on bacterial growth per se to determine whether a substrate has been used. They employ a heavy inoculum in which specific bacterial enzymes are present in amounts sufficient to convert substrate to product rapidly. In addition, some systems use fluorogenic substrate/end-product detection methods to increase sensitivity (through signal amplification).

GAS-LIQUID CHROMATOGRAPHY

Gas-liquid chromatography is often used to detect metabolic end products of bacterial fermentations. One common application is identification of short-chain fatty acids produced by obligate anaerobes during glucose fermentation. Because the types and relative concentrations of volatile acids differ among the various genera and species that make up this group of organisms, such information serves as a metabolic “fingerprint” for a particular isolate.

Gas-liquid chromatography can be coupled to a sophisticated signal-analysis software system for identification and quantitation of long-chain fatty acids (LCFAs) in the outer membranes and cell walls of bacteria and fungi. For any given species, the types and relative concentrations of LCFAs are distinctive enough to allow differentiation even from closely related species. An organism may be identified definitively within a few hours after detection of growth on appropriate media. LCFA analysis is one of the most advanced procedures currently available for phenotypic characterization.

NUCLEIC ACID TESTS

Techniques for the detection and quantitation of specific DNA and RNA base sequences in clinical specimens have become powerful tools for the diagnosis of bacterial, viral, parasitic, and fungal infections. Nucleic acid tests are used for four purposes. First, they are used to detect, and sometimes to quantify, specific pathogens in clinical specimens. Second, such tests are used for identification of organisms (usually bacteria) that are difficult to identify by conventional methods. Third, nucleic acid tests are used to determine whether two or more isolates of the same pathogen are closely related (belonging to the same “clone” or “strain”). Last, nucleic acid tests are used to predict the sensitivity of organisms (typically viruses) to chemotherapeutic agents. Current technology encompasses a wide array of methods for amplification and signal detection, some of which have been approved by the U.S. Food and Drug Administration (FDA) for clinical diagnosis.

Use of nucleic acid tests generally involves lysis of intact cells or viruses and denaturation of the DNA or RNA to render it single-stranded. Probe(s) or primer(s) complementary to the pathogen-specific target sequence may be hybridized to the target sequence in a solution or on a solid support, depending on the system employed. In situ hybridization of a probe to a target is also possible and allows the use of probes with agents present in tissue specimens. Once the probe(s) or primer(s) have been hybridized to the target (biologic signal), a variety of strategies may be employed to detect, amplify, and/or quantify the target-probe complex (Fig. e14-3).

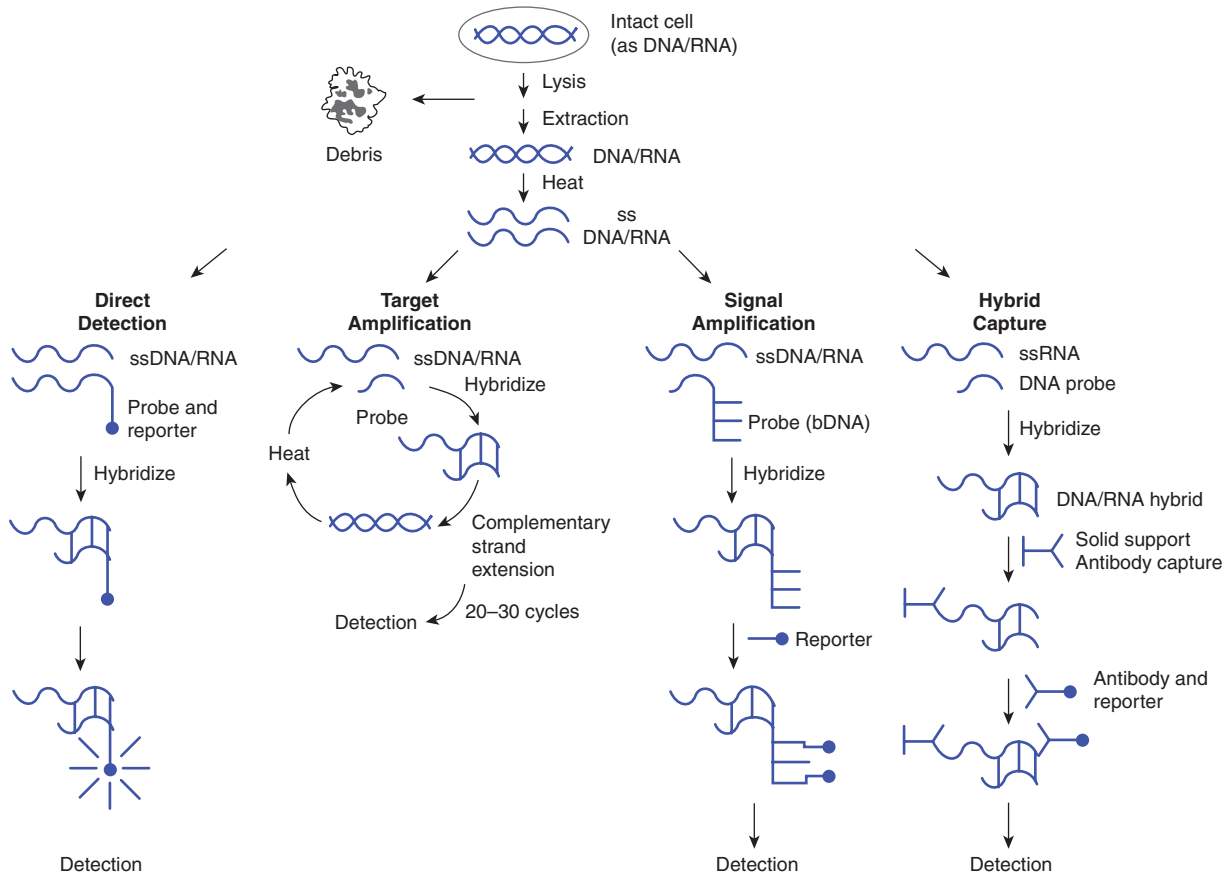


FIGURE e14-3 Strategies for amplification and/or detection of a target-probe complex. DNA or RNA extracted from microorganisms is heated to create single-stranded (ss) DNA/RNA containing appropriate target sequences. These target sequences may be hybridized directly (direct detection) with probes attached to reporter molecules; they may be amplified by repetitive cycles of complementary strand extension (polymerase chain reaction) before attachment of a reporter

Probes for Direct Detection of Pathogens in Clinical Specimens Nucleic acid probes are used for direct detection of pathogens in clinical specimens without amplification of the target strand of DNA or RNA. Such tests detect a relatively short sequence of bases specific for a particular pathogen on single-stranded DNA or RNA by hybridization of a complementary sequence of bases (probe) coupled to a “reporter” system that serves as the signal for detection. Nucleic acid probes are available commercially for direct detection of various bacterial and parasitic pathogens, including *Chlamydia trachomatis*, *N. gonorrhoeae*, and group A *Streptococcus*. A combined assay to detect and differentiate agents of vaginitis/vaginosis (*Gardnerella vaginalis*, *Trichomonas vaginalis*, and *Candida* spp.) has also been approved. An assortment of probes are available for confirming the identity of cultured pathogens, including some dimorphic molds, *Mycobacterium* spp., and other bacteria (e.g., *Campylobacter* spp., *Streptococcus* spp., and *Staphylococcus aureus*). Probes for the direct detection of bacterial pathogens are often aimed at highly conserved 16S ribosomal RNA sequences, of which there are many more copies than there are of any single genomic DNA sequence in a bacterial cell. The sensitivity and specificity of probe assays for direct detection are comparable to those of more traditional assays, including EIA and culture.

In an alternative probe assay, called *hybrid capture*, an RNA probe anneals to a DNA target, and the resulting DNA/RNA hybrid is captured on a solid support by antibody specific for DNA/RNA hybrids (concentration/amplification) and detected by chemiluminescent-labeled antibody specific for DNA/RNA hybrids. Hybrid capture as-

says are available for *C. trachomatis*, *N. gonorrhoeae*, cytomegalovirus, and human papillomavirus. Many laboratories have developed their own probes for pathogens; however, unless a method-validation protocol for diagnostic testing has been performed, federal law in the United States restricts the use of such probes to research.

probe; or the original target-probe signal may be amplified via hybridization with an additional probe containing multiple copies of a secondary reporter target sequence (branched-chain DNA, or bDNA). DNA/RNA hybrids can also be “captured” on a solid support (hybrid capture), with antibody directed at the DNA/RNA hybrids used to concentrate them and a second antibody coupled to a reporter molecule attached to the captured hybrid.

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Nucleic Acid Amplification Test Strategies In theory, a single target nucleic acid sequence can be amplified to detectable levels. There are several strategies for nucleic acid amplification tests (NAATs), including PCR, LCR, strand displacement amplification, and self-sustaining sequence replication. In each case, exponential amplification of a pathogen-specific DNA or RNA sequence depends on primers that anneal to the target sequence. The amplified nucleic acid can be detected after the reaction is complete or (in *real-time* detection) as amplification proceeds. PCR, the first and still most common NAAT, requires repeated heating of the DNA to separate the two complementary strands of the double helix, hybridization of a primer sequence to the appropriate target sequence, target amplification using the PCR for complementary strand extension, and signal detection via a labeled probe. Methods for the monitoring of PCR after each amplification cycle—via either incorporation of fluorescent dyes into the DNA during primer extension or use of fluorescent probes capable of fluorescence resonance energy transfer—have now decreased the period required to detect a specific target. The sensitivity of NAATs is far greater than that of traditional assay methods such as culture. However, the care with which the assays are performed is important, because

e104 cross-contamination of clinical material with DNA or RNA from other sources (even at low levels) can cause false-positive results. An alternative NAAT employs transcription-mediated amplification, in which an RNA target sequence is converted to DNA, which is then exponentially transcribed into RNA target. The advantage of this method is that only a single heating/annealing step is required for amplification. At present, amplification assays for *Mycobacterium tuberculosis*, *N. gonorrhoeae*, *C. trachomatis*, *Mycoplasma hominis*, group B *Streptococcus*, and methicillin-resistant *S. aureus* are on the market. Again, many laboratories have used commercially available *taq* polymerase, probe sequences, and analyte-specific reagents (ASRs) to develop “in-house” assays for diagnostic use. Issues related to quality control, interpretation of results, sample processing, and regulatory requirements have slowed the commercial development of many diagnostic assay kits.

Identification of otherwise difficult-to-identify bacteria involves an initial amplification of a highly conserved region of 16S rDNA by PCR. Automated sequencing of several hundred bases is then performed, and the sequence information is compared with large databases containing sequence information for thousands of different organisms. While 16S sequencing is not as rapid as other methods and is still relatively expensive for routine use in the clinical microbiology laboratory, it is becoming the definitive method for identification of unusual or difficult-to-cultivate organisms.

Quantitative Nucleic Acid Test Strategies With the advent of newer therapeutic regimens for HIV-associated disease, cytomegalovirus infection, and hepatitis B and C virus infections, the response to therapy has been monitored by determining both genotype and “viral load” at various times after treatment initiation. Quantitative NAATs are available for HIV (PCR), cytomegalovirus (PCR), hepatitis B (PCR), and hepatitis C (PCR and transcription-mediated amplification, or TMA). Many laboratories have validated and perform quantitative assays for these and other pathogens (e.g., Epstein-Barr virus), using ASRs for NAATs.

Branched-chain DNA (bDNA) testing is an alternative to NAATs for quantitative nucleic acid testing. In such testing, bDNA attaches to a site different from the target-binding sequence of the original probe. Chemiluminescent-labeled oligonucleotides can then bind to multiple repeating sequences on the bDNA. The amplified bDNA signal is detected by chemiluminescence. bDNA assays for viral load of HIV, hepatitis B virus, and hepatitis C virus have been approved by the FDA. The advantage of bDNA over PCR is that only a single heating/annealing step is required to hybridize the target-binding probe to the target sequence for amplification.

Application of Nucleic Acid Tests In addition to the applications already discussed, nucleic acid tests are used to detect and identify difficult-to-grow or noncultivable bacterial pathogens, such as *Mycobacterium*, *Legionella*, *Ehrlichia*, *Rickettsia*, *Babesia*, *Borrelia*, and *Tropheryma whippelii*. In addition, methods for rapid detection of agents of public health concern, such as *Bacillus anthracis*, smallpox virus, and *Yersinia pestis*, have been developed.

Nucleic acid tests are also used to determine how close the relationship is among different isolates of the same species of pathogen. The demonstration that bacteria of a single clone have infected multiple patients in the context of a possible means of transmission (e.g., a health care provider) offers confirmatory evidence for an outbreak. Pulse-field gel electrophoresis remains the usual gold standard for bacterial strain analysis. This method involves the use of restriction enzymes that recognize rare sequences of nucleotides to digest the bacterial DNA, resulting in large DNA fragments. These fragments are separated by gel electrophoresis with variable polarity of the electrophoretic current and then are visualized. Similar band patterns (i.e., differences in ≤ 3 bands) suggest that different bacterial isolates are closely related, or clonal. Simpler methods of strain typing include sequencing of single or multiple genes and PCR-based amplification of repetitive DNA sequences in the bacterial chromosome.

Future applications of nucleic acid testing will likely include the replacement of culture for identification of many pathogens with solid-

state DNA/RNA chip technology, in which thousands of unique nucleic acid sequences can be detected on a single silicon chip.

SUSCEPTIBILITY TESTING OF BACTERIA

A principal responsibility of the clinical microbiology laboratory is to determine which antimicrobial agents inhibit a specific bacterial isolate. Such testing is used to screen for infection control problems, such as methicillin-resistant *S. aureus* or vancomycin-resistant *Enterococcus faecium*. Two approaches are useful. The first is a qualitative assessment of susceptibility, with responses categorized as susceptible, resistant, or intermediate. This approach can involve either the placement of paper disks containing antibiotics on an agar surface inoculated with the bacterial strain to be tested (Kirby-Bauer or disk/agar diffusion method), with measurement of the zones of growth inhibition following incubation, or the use of broth cultures containing a set concentration of antibiotic (breakpoint method). These methods have been carefully calibrated against quantitative methods and clinical experience with each antibiotic, and zones of inhibition and breakpoints have been calculated on a species-by-species basis.

The second approach is to inoculate the test strain of bacteria into a series of broth cultures (or agar plates) with increasing concentrations of antibiotic. The lowest concentration of antibiotic that inhibits visual microbial growth in this test system is known as the *minimum inhibitory concentration* (MIC). If tubes in which no growth is seen are subcultured, the minimum concentration of antibiotic required to kill 99.9% of the starting inoculum can also be determined (*minimum bactericidal concentration*, or MBC). The MIC value can be given a categorical interpretation of susceptible, resistant, or intermediate and so is more widely used than the MBC. Quantitative susceptibility testing by the microbroth dilution technique, a miniaturized version of the broth dilution technique using microwell plates, lends itself to automation and is commonly used in larger clinical laboratories.

A novel version of the disk/agar diffusion method employs a quantitative diffusion gradient, or epsilometer (E-test), and uses an absorbent strip with a known gradient of antibiotic concentrations along its length. When the strip is placed on the surface of an agar plate seeded with a bacterial strain to be tested, antibiotic diffuses into the medium, and bacterial growth is inhibited. The MIC is estimated as the lowest concentration that inhibits visible growth.

For some organisms, such as obligate anaerobes, routine susceptibility testing generally is not performed because of the difficulty of growing the organisms and the predictable sensitivity of most isolates to specific antibiotics.

SUSCEPTIBILITY TESTING OF FUNGAL AGENTS

With the advent of many new agents for treating yeasts and systemic fungal agents, the need for testing of individual isolates for susceptibility to specific antifungal agents has increased. In the past, few laboratories participated in such testing because of a lack of standard methods like those available for testing bacterial agents. However, several systems have now been approved for antifungal susceptibility testing. These methods, which determine the minimal fungicidal concentration (MFC), are similar to the broth microdilution methods used to determine the MIC for bacteria. The E-test method is approved for testing the susceptibility of yeasts to fluconazole, itraconazole, and flucytosine, and disk diffusion can be used to test the susceptibility of *Candida* spp. to fluconazole and voriconazole. Methods for determining the MFC against fungal agents such as *Aspergillus* spp. are technically difficult, and most clinical laboratories refer requests for such testing to reference laboratories.

ANTIVIRAL TESTING

See Chap. 170.

FURTHER READINGS

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