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DIAGNOSTIC MEDICAL MICROBIOLOGY

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INTRODUCTION

Diagnostic medical microbiology deals with the isolation and identification of infectious agents from clinical specimens. It involves laboratory technicians and senior microbiologists in hospitals and public health laboratories. The techniques used in this discipline are not restricted to the medical laboratory. It is frequently essential for public health reasons to determine the presence or absence of infectious or potentially infectious microorganisms from nonclinical materials. The diagnostic techniques applicable here (e.g., foods, drugs, water, etc.) are also discussed in certain "official" and "semi-official" regulatory compendia.[1]

This review will attempt to give a synopsis of an expanding subject. It is hoped that the reader will gain a general appreciation of the techniques involved in the microbiological diagnosis of disease. In addition, this chapter will stress the importance of microbial physiology as it reduced to practice in the identification of microorganisms. It is to be emphasized that direct experience in the clinical or industrial laboratory under the supervision of an experienced diagnostician is the only way to teach the identification of bacteria. A review of this nature is, therefore, an attempt to explain the theoretical basis of the culture media employed, as well as an attempt to describe the microscopic, colonial, and biochemical tests most current in the art. The reader is encouraged to study more advanced texts and articles if he is interested in the more specialized areas of medical mycology, anaerobic bacteriology, antibiotic sensitivity testing, serology, and virological techniques.[2-4]

PATHOGENICITY

Pathogens

Pathogens are defined as disease-producing microorganisms. They are bacterial, mycotic, protozoan, chlamydial, or viral in nature. No one is sure what a pathogen is. Pathogens can lose virulence or become attenuated and become "nonpathogenic". "Nonpathogens" can gain entry in high numbers during states of reduced resistance and produce disease. The diseases that they produce can be transmitted vertically, horizontally, or via a zig-zag route. Organisms can also be transmitted directly from the environment to an individual (coccidiomycosis, Legionaire's disease, food poisoning). Transmission of infection from mother-to-child (e.g., congenital syphilis, herpes type 2) is an example of "vertical" transmission. Contraction of infection by one individual directly from another is an example of "horizontal" transmission (e.g., streptococcal sore throat, V.D., influenza, etc.). "Zig-zag" transmission is a viral or rickettsial phenomenon associated with insects or ticks which serve as intermediaries between man and other mammals or birds (e.g., an arbovirus disease such as Eastern or Western encephalitis).

There are some organisms which can be regarded as (1) "obligate pathogens", organisms that are extremely invasive at a relatively low multiplicity input regardless of age or state of health (e.g., arboviruses, rabies virus, *Treponema pallidum, Neisseria gonorrhoeae, N. meningitis, Coccidioides immitis, Francisella tularensis, Pasteurella pestis* (Yersinia), herpesvirus. Enter their environment and you've got the disease. Organisms regarded as (2) "pathogens" can be isolated very frequently from diseased sites (e.g., *Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli, Proteus, Streptococcus pneumoniae, Candida, Salmonella,* and *Nocardia*. Every laboratory technician recognizes these as the organisms that you are likely to find. Organisms regarded as (3) "opportunistic pathogens"

(e.g., Gram-negative rods such as member of the genus *Serratia, Acinetobacter Pseudomonas, Enterobacter*), are thought to comprise the normal or associated flora or to be part of the inanimate environment. They react to alterations in host resistance and produce disease. In short, the proposition put simply is either:

1. A pathogen is a microorganism that *can* produce disease (parasite-oriented genotypic view).
2. A pathogen is an organism that *has* produced a disease (host-oriented phenotypic view).

Associated Flora

The normal or associated flora has been considered as a latent reservior of potential infection.[5,6] Associated organisms that exist on or in specific body sites are known to possess specific surface organelles (adhesions) which fit as in a lock and key into specific receptor sites on the host cell.[6] Once adhesion occurs, colonization by the associated flora begins. This may even be considered "infection"; when the equilibrium is destroyed and invasion occurs, the normal "flora" has produced a "disease". This newer concept of associated flora as opposed to normal flora implies the potential pathogenicity of every organism residing in or on the human body. There is no evidence to disprove this theory except, perhaps, that we know that the oral, enteric, and vaginal lactobacilli do not produce infections in man. The key to pathogenicity is attachment, invasiness, and multiplication. Physiological and structural defenses develop with and are a part of the human animal from birth. This defense against invasion begins to falter throughout life; during injury, during concurrent disease, during drug therapy, during old age, and culminates with death. Health lies somewhere amidst the skirmishes of disease, the retreat of obligate pathogens, and the containment of opportunistic associated flora. It is a constant war.

Infectious diseases, therefore, represent a complicated interaction between host and parasite. Accordingly, the medical microbiologist should have a basic knowledge of human anatomy and physiology, immunology and pathology, as well as a knowledge of the "normal" and "abnormal" flora. In this interaction the nature of the parasite may be fixed, a function of its intrinsic genotype. The nature of the host is variable, depending upon age, previous illness, presence of concurrent disease, and previous exposure to drugs. There might then exist a mosaic of host phenotypic states upon which and into which a sufficient number of virulent microbial genotypes can propagate and cause disease. Interposed between the phenotypic fluidity of the host and the genotypic rigidity of the microorganisms is the clinical microbiologist. He must help the physician to diagnose disease or he must help the industrial or public health laboratory to prevent disease.

A monograph on diagnostic microbiology can be indexed according to disease, organism, or specimen. Since the disease or organism is frequently in question, the arrangement of procedures and tests on the basis of specimen is most common and most useful.

A disease-oriented index would cover such topics as shown in Tables 1 and 2. The techniques employed in identifying bacteria related to these diseases rely heavily upon cytology, colonial morphology, microbial physiology, nutritional requirements, and selective inhibition by antimicrobial agents. Advances in our knowledge of metabolic pathways and mechanisms of electron transport have allowed the development of tests that were nonexistent a few years ago. New tests are continually being developed.

Useful Cytological Characteristics

In some poorly supervised laboratories microscopic examinations are totally replaced by biochemical tests. To prevent errors, simple and special staining techniques routinely should

Table 1
CATEGORIES OF DISEASE FOR WHICH A MICROBIOLOGICAL DIAGNOSIS IS REQUESTED

Target Organ Index

Disease: body system	Types	Examples of syndrome
Respiratory	Upper respiratory, lower respiratory, bacterial, viral, fungal	"Cold", sore throat, bronchitis, pneumonia, TB, histoplasmosis, candidiasis
Ophthalmic	Acute bacterial, chronic bacterial, viral, or chlamydial	Conjunctivitis, blephlaritis, herpes (type I), keratitis, APC fever, pink eye, trachoma
Otic	Otitis externa, otitis media bacterial fungal	Swimmer's ear, common ear ache
Febrile systemic	Bacteremia, virema	Scarlet fever, septicemias, meningitis, viral fever and rashes
Gastrointestinal	Bacterial, viral, fungal, acute, chronic	Diarrhea, vomiting, typhoid fever, amebic dysentery, general enteritis, viral fevers and rashes
Venereal	Bacterial, viral, chlamydial, protozoan	Syphilis, gonorrhea, chancroid, herpes type II, (genitalis), lymphogranuloma venereum
Genitourinary	Bacterial, yeasts, protozoan	Urethritis, vaginitis, epididymitis, cystitis, cervicitis, prostatitis
Dermatologic	Bacterial, fungal, viral	Acne, boils, warts, wound infection, dermatomycoses, (athletes foot, ring worm, T. cruris), smallpox, herpes

Table 2
CATEGORIES OF ORGANISMS ASSOCIATED WITH COMMON INFECTIOUS DISEASES

Etiological Index

Organism	Disease
Gram-positive cocci	Pyogenic eye-ear-nose infections, skin infections, "strep" throats, meningitis, boils wound infections, subacute bacterial endocartitis, urethritis, nephritis, prostatitis, septicemia
Acid fast bacilli	Tuberculosis, leprosy (Hansen's disease)
Gram-negative rods	Urethritis, vaginitis, cystitis, nephritis, otitis media, septicemia, diarrhea, appendicitis, peritonitis, burn infections, typhoid fever, dysentery
Gram-positive spore-forming bacilli (a) aerobic (b) anaerobic (c) Gram-positive anaerobic diphtheroids	(a) Anthrax, diarrhea (Bacillus cereus), conjunctivitis (Bacillus subtilis) (b) tetanus, gas gangrene, wound infections, botulism (c) acne (*Propionibacterium acnes*)
Yeasts and molds	Meningitis, aspergillosis, histoplasmosis, vaginitis, otitis externa, coccidioidomycosis, cryptococcosis, candidiasis, nail and skin infections (onychomycosis and dermatomycosis)
Viruses	DNA vs RNA; ether sensitive vs. ether resistant; polio, diarrhea, fever and rash, influenza, measles, herpes (eye or genitals), mumps, gastritis, common cold, rabies, meningitis, smallpox, myocarditis, enteritis, Bornholm's disease, APC fever (adenovirus syndrome), infectious mononucleosis (E-B virus).
Gram-negative cocci	Conjunctivitis, gonorrhea, meningitis
Gram-positive nonsporulating rods	Actinomycosis (cutaneous and systemic); nocardiosis (cutaneous and pulmonary)

Note: Table 2 is designed to show, from a different perspective, diseases as they are associated with specific groups of organisms, and is, accordingly, an etiology-oriented index.

be employed. For example, an entire battery of tests for pathogenic enteric Gram-negative rods may be needlessly performed on a harmless spore-forming member of the genus *Bacillus*. To avoid this, Gram stains should be performed on all atypical or sparse growth on EMB agar to rule out the presence of Gram-positive bacilli. It is a common misconception that EMB only supports the growth of Gram-negative bacteria. *Hemophilus hemolyticus* can be misdiagnosed as *Streptococcus pyogenes* (both β-hemolytic) by failure to perform a Gram stain on all small-sized colonies detected on blood agar. Similarly, confusion between a diagnosis of *Enterobacter* or *Klebsiella* can be resolved by a test for motility. The microscope is frequently the most under-used piece of equipment in the clinical laboratory. The most useful tests are (wet mount or stain):

1. Shape (rods, spheres, chains, clusters, filamentous, branching)
2. Gram reaction (proper attention to age and source of culture or specimen)
3. Acid fast reaction
4. Spore formation (endospores for bacteria, conidia and chlamydospores for fungi)
5. Motility
6. Type of flagellation

Morphology is especially important in the identification of fungi and actinomycetes. These should be observed in undisturbed cultures in order to detect the formation and arrangements of conidia and other sporulating structures. True fungi and actinomycetes are identified by the microscopic appearance of spores (conidia) and the spore-bearing structures (conidiophores). Dermatophytic fungi are identified by the size of the spores (microconidia vs. macroconidia) as well as by hyphal structure (racquet hyphae, spiral hyphae, pectinate bodies). It is important to recognize that the carbohydrate or nitrogen content of the medium can affect spore production.

Some Useful Biochemical Reactions

Biochemial reactions elicited by organisms grown on specific substrates can be determined by a variety of conventional test media or by more recently developed multiple-test rapid diagnostic kits. They are especially useful in the identification of Gram-negative rods (fermenters and nonfermentors). Rapid diagnostic kits are also available for yeasts and staphylococci. A partial list of biochemical reactions used as phenotypic traits appears below:

1. Production of acid and gas from glucose and other hexoses*
2. Cleavage of disaccharides and fermentation of corresponding hexoses
3. Oxidation of hexoses
4. Oxidation of disaccharides
5. Amino acid deaminases and decarboxylases
6. Cytochrome oxidase
7. Catalase production
8. Production of H_2S
9. Production of indole from tryptophane
10. Dissimilation of glucose to acetyl methyl carbinol (Voges-Proskauer test)
11. Utilization of citrate as a sole source of carbon
12. Coagulase production

* The acids detected through phenol red or brom thymol blue pH changes are usually lactic, pyruvic, acetic, or formic acid, or combinations of these. If an organism produces stoichiometric amounts of lactic acid from glucose, it is called "homofermentative". If it produces a mixture of acids, is it called "heterofermentative". The gases produced are for the most part CO_2, H_2, or a mixture of these. Oxidative organisms generally produce weaker organic acids such as gluconic or 2-keto-gluconic acid.

Table 3
SELECTIVE INHIBITORS IN CULTURE MEDIA

Inhibitor	Organisms inhibited
KCN	Certain GNR and GP
Boric Acid	Certain GNR, GPC, GPB at 43C
Na lauryl sulfate	GPB, GPC
Na azide	GNR
Crystal violet	Gram positive
Na desoxycholate	Gram positive
B-Phenethylalcohol	GNR
Cetyl trimethyl ammonium bromide (cetrimide)	GNR, except *Pseudomonas* and *Serratia* and some *Enterobacter*, GPC, GBP
Brilliant green	GPC, GPB
NaCl	GNR, Streptococci (concentration-dependant)
Neomycin SO_4	General bacteria as required
Sodium sulfite and basic fuchsin	GP
Eosin Y, methylene blue	GP
Chloramphenicol	General bacteria as required
Malachite green	General bacteria as required (TB culture)
Cyclohexamide	Nonpathogenic fungi
Na selenite	Certain GNR (e.g., coliforms)
Na Heptadecyl SO_4 (Tergitol)	GPC, GPB
Nitrothiazole acetyl piperazine or nitroimidazoles	Anaerobes
Tetracycline	General bacteria as required

Note: GNR, Gram-negative rods;
GP, Gram-positive (C) cocci, (B) or bacilli.

Some Useful Inhibitory Media

Inhibitory media are essential when working with a mixed flora. Such media are described as "selective" or "differential". In most cases, an inhibitory medium is selective and differential with respect to occurrence and appearance of growth. Selective media are designed to encourage the growth of certain organisms and to inhibit the growth of others (e.g., general taxons such as Gram-positive vs. Gram-negative bacteria), or to allow visual discrimination among colony types within on generally selected taxon (e.g., *Salmonella* vs. *Escherichia*). Since most clinical specimens contain a mixed flora, selective and differential media are routinely employed.

The incorporation of antimicrobial agents into culture media is essential for the selective growth of organisms from a mixed population. In the early part of the 20th century microbiologists empirically tested the effect of natural substances and dyes. In more recent times antibiotic and/or synthetic chemicals have been added to broth or agar to shift a mixed flora in the direction of growth of a single taxon or group of bacteria. Table 3 gives examples of some of the more useful agents currently employed in clinical microbiology. As can be noted, basic dyes, desoxycholic acid, antibiotics, and inorganic salts are effective agents. Most of these antimicrobial agents were detected by chance or empirical screening.

Media used in diagnostic bacteriology are designed to support and/or selectively inhibit growth with production of a visual effect. The visual effect is detected by incorporation of pH indicators in the medium or is developed by the addition of colorimetric reagents to the culture tubes. The biochemical reactions found useful are predicated upon the detection of threshold amounts in assays with limited sensitivity. Lysine decarboxylase is significant in the detection of *Salmonella* but one must not assume that lysine decarboxylase is absent in *Escherichia coli*. Cytochrome oxidase is significant for *Pseudomonas* but one must not assume that it is absent in *Acinetobacter*. It is a question of degree and not an absolute

presence or absence of any of these enzymes. In clinical medicine, a more quantitative assessment is made within a range of normal values (e.g., alkaline phosphatase, blood glucose, glutamine-oxalacetate transaminase, etc.). The point is that the technician performing blood chemistries on humans and the technician performing biochemical tests on cultures are studying the same thing, outlying values detectable by quantitative tests in humans or quick "go-no-go" qualitative tests in bacteria.

As the microbiologist gains experience, prudent use of a few selective and differential media will give rapid presumptive answers as to genus, so as to quickly aid the physician (see Table 4). Extensive biochemical tests are required for definitive speciations. The exigencies of patient care, however, frequently place a greater premium on determinations of genus and antibiotic sensitivity than on complete identification. It is important to identify the source of the organism and/or the suspected disease so that the microbiologist can gain an early diagnostic lead.

Table 4 is a practical guide to the diagnostician insofar as it suggests specific culture media for specific specimens. At the same time it gives a synopsis of the organisms to be expected and the diseases for which a diagnosis is being made. The great majority of these culture media are available commercially and require little time to prepare. Differences exist with respect to stability, storage, and the need to add supplements, such as vitamin mixtures, carbohydrates, serum, blood, etc. The diagnostic microbiologist should be aware of the basic composition of all culture media employed as well as be aware of some of the theoretical considerations which allow the components of the medium to differentiate and select.

Inoculation of Specimens

All specimens received in the laboratory should be properly identified with the patient's name, a number, source of specimen, date of specimen, attending physician, and tests required, and then promptly inoculated. Samples can be refrigerated, but the time should, in general, not exceed 6 hr (24 to 48 hr for urine). Ancillary information on clinical diagnosis or current antibacterial therapy is often helpful. All samples, except stools, should be collected in a sterile container. Environmental samples (floors, water, equipment) should be identified so as to exclude the use of inappropriate diagnostic media.

The choice of media for primary isolation and the decision to perform or not perform a Gram stain on the specimen is important. The chief objective of a clinical microbiology laboratory is to recover organisms and then to accurately identify them. Accordingly, the type of media and the method of inoculation will determine to a large extent the success of the laboratory in making a bacteriological diagnosis (see Table 4). The use of Gram stains, enrichment tests, differential or selective media depends upon a variety of factors, not the least of which is the speed with which a diagnosis is desired. In certain cases, Gram stains and all three types of media are used together. Selective media are the most inhibitory and care should be exercised in attempting primary isolations in the presence of the various antimicrobial agents. Some of the more common specimens will be described along with the primary methods of inoculation and the organisms most likely to be encountered.

Normally Sterile Sites

Specimens from normally sterile sites should be cultured on media capable of supporting the growth of any organism likely to be present, including the most fastidious pathogens. Differential and/or selective media should never be used alone. Any organism isolated from blood, bone marrow, tissue, cerebrospinal, synovial, pleural, or ascitic fluid should be carefully evaluated. An organism normally regarded as "nonpathogenic" should be evaluated in the light of the numbers present and the consistency of isolation before being discarded as a contaminant. The final decision is that of the attending physician. *Staphylococcus epidermidis* is of no significance on the skin but is significant in the blood.

Table 4
SPECIMEN GUIDE TO CHOICE OF MEDIA

Specimen	Source	Common organisms suspected	Disease	Some culture media
Throat, nose, ear or eye swab	Pharynx or nasopharynx or draining ear	*S. aureus, S. pyogenes, H. influenza, S. pneumonia,* meningococcus, *C. albicans,* myxoviruses, rhinoviruses	Pharyngitis, sinusitis, thrush, sore throat, otitis media	Blood agar under 10% CO_2 and anaerobic, gram stain, Choc. agar TSI, M-S
Sputum	Lower respiratory tract (not saliva) (1st am specimen)	*S. aureus, Klebsiella, S. pyogenes, S. pneumonia, M. tuberculosis, Histoplasma, C. albicans, Cryptococcus neoformans*	Various pneumonias, tracheitis, tuberculosis, various mycoses, bronchitis, bronchiolitis	As above (special treatment for TB)
Urine	AM, 3-glass specs, catheterization or midstream	*S. aureus, E. coli, Klebsiella Ent./Serratia, S. pyogenes, M. tuberculosis, Ps. sp., Proteus*	Cystitis, nephritis, urethritis, vaginitis, prostatitis	Blood agar, Mac, EMB, M-S quantitative loop onto plates or broth dilution for bacteriuria, TSI
Blood	Venous	A variety of aerobic or anaerobic GP and GN organisms	Febrile disease, subacute bacterial endocarditis, etc.	TSB, BHI (aerobic and anaerobic) FTM
Gastrointestinal	Feces	*S. aureus, E. coli, Salmonella arizona, Shigella, Cholera vibrio, B. cereus, C. perfingers, Vibrio parahemolyticus*	Typhoid fever, dysentery, diarrhea, enteritis, "food poisoning", gastritis	Selenite cysteine, EMB lactose, Mac, XLD, TSI, FTM, Blood agar
Uro-genital swabs	Urethral exudate Vaginal exudate Prostatic secretion Cervical swab	*Staph., Strp., Hemophilus, (corynebacterium) Candida, E. coli, gonococcus, Trichomonas, Proteus, Pseudomonas*	Urethritis, vaginitis prostatitis, vulvitis	Blood agar, Thayer-Martin CO_2, Choc. Mac FTM, cooked meat if anaerobes suspected EMB, FTM
Wounds	Surgical specimens, tissues, skin swabs, etc.	A variety of aerobic and anaerobic GP and GN organisms	Superficial or deep infection	Blood agar, CO_2 anaerobic, MAC, M-S, cooked meat

Note: TSI, triple sugar iron agar; M-S, mannitol salts agar; FTM, fluid thioglycollate medium; GN Gram negative; EMB Eosin methylene blue agar; MAC, MacConkeys agar; XLD, xylose lysine decarboxylase agar; GNR, Gram-negative rod (s); LIA, lysine iron agar; SS, Salmonella Shigella; GP, Gram-positive; BHI, brain-heart infusion; Choc., Chocolate agar.

Heavy Normal Flora Sites

Specimens from the skin and various body orifices are cultured as above but differential and selective media are also employed to prevent overgrowth of organisms normally present (e.g., chloramphenicol to inhibit skin bacteria in isolating dermatophytes). The question of what is a contaminant and what is a potential pathogen is a difficult one when dealing with heavily contaminated sites. The bacteriologist, from his retrospective knowledge of the "normal" or associated "flora" (e.g., throat or skin or vagina), must decide whether a shift to a predominant flora has occurred. This would indicate that the relative balance of the normal flora has been altered in favor of a potential pathogen. Specimens from the throat, lungs, and intestinal tract pose special problems. Under normal conditions certain organisms are not pathogens; under special circumstances they can produce infections (phenotypic vs. genotypic view). Unfortunately, the bacteriologist does not have a "normal flora" reference for a specific patient as a cardiologist has a prior EKG, or a radiologist has a prior lung X-ray. A "heavy normal" flora should be reported, as it may indirectly indicate a viral infection which has compromised the normal host tissue and altered the resident ecological balance. Organisms that might be encountered from sites of heavy resident flora are: staphylococci, fermenting and nonfermenting Gram-negative rods, streptococci, members of the genus *Neisseria*, diphtheroids, lactobacilli, and a variety of Gram-positive and Gram-negative anaerobes.

Delivery of Specimen to the Lab

Specimens are most frequently inoculated to solid or broth medium by the use of swabs. Swabs that must be sent through the mail should be coated with a nonnutritive protective medium such as Stuart's transport medium, where viability at room temperature can be extended. Fluids are best inoculated with a bacteriological loop instead of a swab since small numbers of cells may be absorbed to the fiber and not be recovered. In some cases, a sterile Pasteur pipette or calibrated loop can be used.

Tissue specimens from biopsy or autopsy should be minced in saline in a sterile mortar and pestle to give a 10 to 20% suspension. All combinations of primary, differential, and selective media should be inoculated, since it is seldom possible to obtain a second specimen. The homogenate should be inoculated by loop or pipette as with a fluid.

EENT Specimens — Eye, Ear, Nasal, Throat, and Pharyngeal Swabs and Exudates

The material submitted to the laboratory is streaked onto blood agar and incubated under 10% CO_2 (candle jar) and in a anaerobic jar to aid in the isolation of beta-hemolytic streptococci. In addition, inoculate plates of EMB and MacConkey's agar for the detection of Gram-negative rods and tubes of FTM to detect anaerobes. If *Hemophilus influenza* is suspected either clinically or as a result of Gram stain, rabbit blood agar is preferred since sheep blood may inhibit *Hemophilus* species. The hemolytic reactions of streptococci on blood agar are described later in this chapter. Differentiation of beta-hemolytic streptococci *(Streptococcus pyogenes)* from *Hemophilus hemolyticus* is made by Gram stain and by failure to grow well on sheep blood agar. Differentiation of hemolytic streptococci from beta-hemolytic staphylococci is made on the basis of colonial morphology, pigment, and by the catalase test, one of the simplest biochemical tests performed by the diagnostic microbiologist. Add a drop of 3% H_2O_2 to some growth rubbed onto the surface of a microscope slide or add 0.5 mℓ of 3% H_2O_2 to 0.5 mℓ of a broth culture. Staphylococci but not streptococci cause evolution of oxygen. This test is also useful in differentiating between nonhemolytic streptococci and catalase positive nonhemolytic diphtheroid organisms, which are frequently part of the upper respiratory flora. The principle strains of *Streptococcus* producing communicable disease in man belong to Lancefield's Group A (pharyngitis, rhinitis, tonsillitis, pneumonia, scarlet fever, as well as pyodermas and septicemia). Sus-

ceptibility to the antibiotic bacitracin, 0.04 unit (disc), helps differentiate type A from beta-hemolytic B,C,D,F, and G, the latter serological types being insensitive to this antibiotic.

Streptococcus pyogenes colonies at 18 to 24 hr on blood agar (reduced O_2 tension) are about 0.5 mm in diameter, transparent to opaque and domed with a smooth surface and entire edge. The organism is virulent for albino mice, 0.5 mℓ intraperitoneally of a 10^{-3} dilution of an overnight broth culture killing mice in 24 to 48 hr.

Streptococcus pneumoniae (formerly *Diplococcus pneumoniae*) produces alpha-hemolysis of sheep blood agar and is also extremely virulent for white mice by the i.p. route and for rabbits s.c. This organism is found in 30 to 70% of normal humans and from this site can invade the sinuses or middle ear (otitis media), eventually producing mastoiditis, meningitis, purulent conjunctivitis, or pneumonia. Colonies on blood agar under CO_2 are alpha-hemolytic, round with entire edges, domed, mucoid in appearance, and about 1 mm in diameter. Anaerobically the colonies show beta-hemolysis. Differentiation from other alpha- and beta-hemolytic organisms can be made on the basis of the bile solubility test (flood the plate with a solution of 10% Na desoxycholate; colonies of the pneumonococcus will dissolve). Pneumococci are also sensitive to the ethylhrocuprein hydrochloride (EHC) and will show a zone of inhibition around a 5 mcg paper disc containing this diagnostic agent.

5. Genitourinary Tract (GU) Specimens — Exudates and Urine

The detection by pour plates, tube dilution, or surface streaking of a calibarated loop of 100,000/mℓ or more organisms in a clean-catch midstream sample or in a catherized specimen is considered a significant bacteriuria. Direct surface streaking with a 0.001-mℓ loop onto MacConkey's agar allows more rapid enumeration of Gram-negative forms (cystitis, nephritis, pyelitis). Isolated colonies can be picked directly to TSI slants and speciations rendered as per the appropriate biochemical tests. The physician should be kept closely informed of the numbers and types isolated.

Urethreal and vaginal exudates or cervical swabs should be Gram stained and streaked onto a suitable agar as rapidly as possible (sheep blood (CO_2) agar, MacConkey's agar, Chocolate agar (CO_2) and a plate of Thayer-Martin (CO_2) medium if the gonococcus is suspected). Incubate all plates at 35°C and examine at 24 and 48 hr. Examine for staphylococci, streptococci, anaerobes, and Gram-negative rods as described above for EENT and GU cultures.

SPECIAL TESTS FOR GRAM-POSITIVE COCCI

Test	Staph aureus	Staph epidermidis	*Micrococcus*	*Strept. pneumoniae*	Beta-strep. sp.	Other streptococci	
						Fecal	Other
Hemolysis (Beta)	+	0	0	0	+	0	0
7.5% salt	+	+	0 to var.	0	0	0	0
Mannitol	+	0 to var.	0	0 to var.	0	0	0 to var.
Coagulase	+	0	0	0	0	0	0
DNAase	+	0	0	0	0	0	0
Anaerobic growth	+	+	0	+	+	+	+
Catalase	+	+	+	0	0	0	0
Sensitivity to bacitracin	NA	NA	NA	NA	+	0	0
Bile solubility	0	0	NA	+	0	0	0
Sensitivity (EHC)	NA	NA	NA	+	0	NA	NA

NA = not applicable.

Blood Cultures

When a physician requests a blood culture the technician must appreciate that a critically

ill patient is involved. Extreme care must be exercised at all stages of the analysis. Detection of true bacteremia can only be accomplished under conditions which minimize contamination during collection and by the use of enriched and osmotically stabilized aerobic and anerobic culture media. Osmotic stabilization of organisms having undergone cell wall damage (L-forms) can be achieved by the addition of 10% sucrose to trypticase soy broth, brain-heart infusion broth, or fluid thioglycollate medium. The inclusion of sodium polyanethol sulfonate (Liquoid®) will prevent clotting of the blood and will neutralize the bactericidal effect of human serum and inactivate certain antibiotics. Sodium citrate, 0.5% in any of the above broths is also an effective anticoagulant. Although procedures will vary from hospital to hospital, a generally acceptable technique is to withdraw 10 mℓ of blood and inoculate 5 mℓ into 100 mℓ of supplemented FTM and 5 mℓ into 100 mℓ supplemented TSB. These techniques will allow propagation of aerobic and anaerobic streptococci, pneumonococci, staphylococci, meningococci, coliform bacilli, and members of the genera *Salmonella, Hemophilus, Bacteroides, Clostridium, Acinetobacter, Pseudomonas, Alcaligenes, Candida, Brucella,* and species of *Francisella (Pasteurella tularensis) Yersinia (Pasteurella pestis)* and *Pasteurella multocida.*

Blood cultures are incubated at 35 to 37°C and examined daily for turbidity up to 10 days. When signs of growth appear (turbidity or gas) Gram stains and subcultures should be made on a variety of media (blood agar, chocolate agar, MacConkey's agar, EMB agar) set up in pairs and incubated aerobically and anaerobically. If all cultures, subcultures, and Gram stains are negative, the blood culture may be reported as "no growth detected". If the physician suspects viremia, appropriate inoculations into tissue culture, chick embryos, and suckling mice must be considered, as well as appropriate serological studies on paired acute and convalescent sera (see Table 5).

Viral cultures — A complete discussion of virus isolation and identification is beyond the scope of this chapter. A brief synopsis of appropriate tests is shown in Table 5. Tissue cultures must be supplemented with penicillin and streptomycin and in some cases amphotericin to prevent overgrowth by bacteria and fungi. Neomycin, gentimycin, or other combinations can also be employed. Balanced salt solutions that are used to obtain throat washings or fecal suspensions must also contain appropriate antimicrobial agents. All attempts at viral isolation and identification must be performed by a properly trained and apprenticed virologist.

SPECIFIC CULTURE MEDIA

The list presented here is not exhaustive, but attempts to portray typical reductions of theory to practice by describing the chemical composition and the microbial reactions on media frequently used in the diagnostic laboratory. In all cases, the ingredients are listed in grams per liter of reconstituted broth. Most of those media are commercially available (DIFCO, BBL, etc.) and are easily reconstituted with deionized or distilled water. After preparation, all media should be quality controlled by challenge with appropriate sensitive as well as responsive organisms. All of the media listed can be obtained from the commercial sources cited in the references.[7,8]

Blood Agar: pH 7.3 ±

Any all-purpose medium such as nutrient agar, brain heart infusion agar, or soy-bean casein digest agar to which 5% defibrinated blood has been added to is called blood agar. The most commonly employed supplement is whole citrated or defibrinated sheep blood. Goat, horse, or rabbit blood may be employed. Human blood can be employed but care must be taken to test for the presence of streptococcal antistreptolysin antibodies. The secretion of extracellular hemolysins produces complete lysis of the erythrocytes around the colony (beta-hemolysis), or incomplete hemolysis, as shown by a green discoloration of the

Table 5
CLINICAL ISOLATION AND IDENTIFICATION OF VIRUSES

Virus	Clinical material	Susceptible host	Cell culture and response	Other tests	Type blood for HI
Adeno	Eye, throat swab	H. Ep 2, HEK, HeLa	CPE (rounded cells)	SNT-CC, HI, FA, CF	Rat or rhesus
Arbo	Autopsy, serum, CSF	S. mice(death), duck embryo	Plaques-BHK and vero cells	SNT-CC mice, CF, HI	Goose
Corona	Throat and nasal	HETOC, HEK, S. mice (death)	Cilia, inhibition, CPE	HI-OC 43, EM, SNT-CC, or mice	Chick
Coxsackie A	Feces and throat	S. mice (death), PMK	Flaccid paralysis, CPE	SNT-mice,SNT-CC	
Coxsackie B	Feces and throat	PMK, WI-38, S. mice (death)	S. mice (death) (spastic paralysis), CPE (R/S/D)	SNT-CC	
Cytomegalo	Urine and throat	HEL	CPE (giant cells, focal lesions)	IHA, CF, FA	Sheep
ECHO	Feces	PMK, HEL	CPE(R/S/D)	SNT-CC	
Epstein Barr	Throat and blood	Lymphoid cells	Experimental transformation	SNT, IFA	
Herpes simplex	Throat and vesicles, buccal, eye, genital	HEL, H.Ep2, WI-38, MRC-5	CAM (Pocks), CPE, S. mice (death)	IHA, CF, FA, SNT-CC	Sheep
Influenza	Nasal and throat	AMN, all eggs, PMK	Amniotic fluid (HA test), HAD on inoculated cell cultures	HI, CF, DID	Guinea pig, chick, human O
LCM	Blood or CSF	3 week mice	BHK cells	FA, Brain smears, SNT-mice	
Measles	Throat and urine	PMK	CPE	SNT-CC, FA, HA	Vervet
Mumps	Throat	PMK	HAd on inoculated cell cultures, syncytial CPE on some cultures	HI, HAdI	Guinea pig
Parainfluenza	Throat	PMK	HAd on inoculated cultures	HI, HAdI	Guinea pig
Polio	Feces and CSF	PMK, WI-38, H.Ep2	CPE	SNT-CC	
Rabies	Brain	3 week mice	Death (IC inoc.)	FA, SNT-mice	
Reo	Throat and feces	PMK, HEK	CPE	SNT, HI	
Respiratory syncytial	Nose and throat	H.Ep2, PMK	CPE	SNT-CC, CF, FA	
Rhino	Nasal and throat	HEL, WI-38	CPE	SNT-CC	
Rubella	Throat	PAGMK, RK-13	No CPE in PAGMK use interference test for ID	Detect. by INT. (PAGMK) SNT-CPE (RK-13)	1 day old chick
Vaccinia	Vesicles	CAM, PMK, WI-38	Pocks on CAM, CPE (rounding, CB)	EM, AGP, FA	Human O, chick
Varicella-Zoster	Vesicles	HEL	CPE	SNT-FA	
Variola	Vesicles	CAM	CPE, Pocks on CAM	EM, AGP, pocks reduction	

Table 5 (continued)
CLINICAL ISOLATION AND IDENTIFICATION OF VIRUSES

Key:

AGP	= agar gel precipitin	HI	= hemagglutination inhibition
ALL	= allantoic cavity	HEK	= human embryonic kidney
AMN	= amniotic cavity	HEL	= human embryonic lung
BSS	= balanced salt solution	HETOCH	= human embryonic tracheal organ culture
BHK	= baby hamster kidney cells	H.Ep 2	= human epithelioma number 2
CB	= cytoplasmic bridging	IFA	= indirect fluorescent antibody
CC	= cell culture	IHA	= indirect hemagglutination
CAM	= chorioallantoic membrane	IC	= intracerebral
CF	= complement fixation	INT	= interference test
CNS	= central nervous system	LCM	= Lymphocytic choriomeningitis
CPE	= cytopathic effect	MIT	= metabolic inhibition test
CSF	= cerebral spinal fluid	OC-43	= coronavirus strain
D. emb.	= duck embryo	PAGMK	= primary African green monkey
DID	= double immunodiffusion	PMK	= primary Rhesus monkey kidney
EM	= electron microscopy	RFIT	= rapid fluorescent focus inhibition test
FA	= fluorescent antibody	RK-13	= rabbit kidney cells
HA	= hemagglutination test	R/S/D	= rounding, shrinking, degeneration
HAd	= hemadsorption	RSV	= respiratory syncytial virus
HAdI	= hemadsorption inhibition	SNT	= serum neutralization test
		S. mice	= suckling mice

hemoglobin around the colonies (alpha-hemolysis). Failure to affect the cellular integrity of erythrocytes, as seen by no change in the color of the agar surrounding of the colony, is also of diagnostic value (gamma-hemolysis). All blood agar should be freshly prepared, preincubated at 35 to 37°C for sterility, and quality control checked with human strains (*Streptococcus pyogenes* for beta-hemolysis, *Streptococcus viridans*, or *Streptococcus pneumoniae* for alpha-hemolysis).

The presence or absence of beta-hemolysis is useful not only for the streptococci and staphylococci but is a useful diagnostic aid for speciations of Gram-negative rods and spore-forming members of the genus *Bacillus* and other groups. Typical hemolytic reactions can be inhibited by the presence of carbohydrates; accordingly, any blood agar base employed must not contain glucose.

Chocolate Agar

Chocolate agar is essentially a blood agar heated to obtain lysis of the erythrocytes and coagulation of the serum proteins. It was originally described early in this century to detect *Hemophilus pertussis*. Whole defribrinated blood alone, or in combination with dehydrated hemoglobin, may be employed, as well as other supplements, e.g., 1% Iso Vitalex® (BBL mixture of vitamins, amino acids, purines, and pyrimidines). Lysing of the blood cells causes the di- and triphosphopyridine nucleotides and heme to become available to fastidious organisms. Chocolate agar is especially useful for the isolation and maintenance of species of *Hemophilus*, *Neisseria* and *Corynebacterium vaginale* (formerly *Hemophilus vaginitis*) (*Gardnerella*). Species of *Staphylococcus* and *Streptococcus* will also grow luxuriantly but the typical hemolytic reactions seen on blood agar will not be noted.

Eosin-Methylene Blue Agar (EMB) pH 7.1 to 7.2

	grams per liter
Peptone	10
Lactose	10
Sucrose	5 (optional*)
Dipotassium phosphate	2.0

Agar	15.0
Eosin Y	0.4
Methylene blue	0.065
Distilled water	1000 mℓ

* When present, lactose is at 5 g/ℓ.

This medium is primarily used to presumptively identify *Escherichia coli*, which produces a blue-black colony with a greenish-metallic sheen. Although differentiation depends upon pH changes, the medium does not contain a conventional pH indicator. The dark color results from fermentation of lactose or sucrose. Nonlactose fermenters produce colorless or high pink colonies *(Salmonella, Shigella, Pseudomonas)*.

The metallic green-black coloration produced by the lactose and/or sucrose fermentations is due to concentration of the insoluble eosin-methylene blue complex precipitated out at low intracolonial pH (similar to the metallic precipitate seen with Wright's stain when staining blood smears). Caution should be exercised in identifying *E. coli* merely on the basis of the metallic sheen, since members of the genera *Enterobacter* and *Citrobacter* can produce the same effect, as can *Proteus* if sucrose is present. Lactose-fermentating members of the *Klebsiella-Enterobacter* group typically produce large mucoid colonies with blue-purple centers. The combination of eosin and methylene blue acts as an inhibitor of Gram-positive organisms. The inhibition is not total, however, and colonies of *Bacillus* and *Staphylococcus* will appear, although small and atypical in size. EMB is also useful in the differentiation of *Candida* species: (1) *C. albicans* = spidery colonies, (2) other *Candida* or yeast = smooth, round colonies.

MacConkey's Agar: pH 7.1 +

	grams per liter
Peptone	17.0
Proteose (polypeptides)	3.0
Lactose	10.0
Bile salts	1.5
NaCl	5.0
Agar	13.5
Neutral red (vital dye)	0.03
Crystal violet	0.001
Distilled water	1000 mℓ

MacConkey's agar shares two things in common with EMB: (1) Gram-negative rods are selected and differentiated with concurrent inhibition of Gram-positive organisms and (2) the diagnostic color changes are related to fermentation of lactose, but are not related to the inclusion of a conventional pH indicator. The medium is more selective than EMB and relies upon a bile salt mixture and crystal violet to inhibit the Gram-positive forms. It is especially useful for the detection of enteric organisms; lactose-fermentating strains produce brick red colonies while lactose-negative forms (*Salmonella, Shigella, Pseudomonas, Acinetobacter, Proteus*) produce pink or colorless colonies. Typical strains of *Escherichia coli* also show a zone of opaque red precipitation around the colony, whereas, other lactose-fermenters will show the red colony with a pale pink periphery and no pericolonial precipitate. As with EMB agar, extremely glycolytic strains may produce intracolonial acidity that concentrates the neutral red dye (red colonies). On this medium, lactose-negative colonies often produce a pale yellow discoloration of the agar. Bacteriuria (10^5 organisms/mℓ in urine) is diagnosed on this medium by direct streak with a calibrated loop.

Endo Agar: pH 7.4 ±

	grams per liter
Dipotassium phosphate	3.5
Peptone	10.0
Agar	15.0
Lactose	10.0
Sodium sulfite	2.5
Basic fuchsin	0.5

Endo agar is a solid medium for the detection of coliform and other enteric forms. It is also used commonly in public health laboratories to detect coliforms in drinking water. Gram-positive bacteria are inhibited by the combination of sodium sulfite and basic fuchsin. Lactose-fermentating bacteria produce pink to red colonies with or without a brick red metallic sheen; as with MacConkey's agar, pericolonial reddening of the agar may occur (but by an entirely different organic reaction). Strains of *Salmonella*, *Shigella*, *Pseudomonas* and other lactose-negative species produce colorless to faint pink colonies. The classical Feulgen reaction (Schiff's reagent) is employed here. Strains such as *E. coli* cleave lactose and ferment glucose with production of acetaldehyde. Acetaldehyde fixes the bisulfite from the decolorized bisulfite-fuchsin reagent (bisulfite adds across the double bond of acetaldehyde) and the regenerated red fuchsin dye concentrates inside the colony.

Cetrimide Agar: pH 7.2 ±

	grams per liter	
Peptone	20.0	supplement with 10 mℓ of glycerol per liter
Magnesium chloride	1.4	
Potassium sulfate	10.0	
Agar	13.6	
Cetrimide*	0.3	
Distilled water	1000 mℓ	

* Cetyl trimethyl ammonium bromide.

This medium is employed for presumptive identification of *Pseudomonas aeruginosa*, which is not inhibited by the quaternary salt (cetyl trimethyl ammonium bromide). The medium promotes the development of the water-soluble pigment pyocyanin as well as the pigment fluorescein, giving the colonies and the surrounding agar a characteristic blue-green color (sometimes reddish or lavender).* Confirmation of *P. aeruginosa* should be made by testing for cytochrome oxidase, reduction of NO_3^- to N_2, growth at 42°C, growth on SS, oxidation but not fermentation of glucose, motility and, ideally, a falgellar stain. Not all strains produce pyocyanin and so this battery of tests should be also run on the nonchromogenic strains (5 to 10%). Other pseudomonads that will grow and produce the typical greenish-yellow water-soluble pigment on cetrimide agar are *P. putida*, *P. fluorescens* (caution: also *Serratia*). *P. maltophilia*, and *P. stutzeri* do not grow on this medium and furthermore, *P. maltophilia* is cytochrome oxidase negative. *Ps. cepacia* produces variable usually negative growth on this medium but is cytochrome oxidase positive. This organism also oxidizes mannitol and lactose and is esculin positive but does not produce DNAase.

* If the pigment pyorubin is formed.

Mannitol Salt Agar: pH 7.4 ±

Beef extract	1.0
Proteose peptone No. 3	10.0
Sodium chloride	75.0
Mannitol	10.0
Agar	15.0
Phenol red	0.025
Distilled water	1000 mℓ

This medium is recommended for the selective isolation of pathogenic staphylococci, which are tolerant of the high-salt concentration. Strains of *S. aureus* that are putatively pathogenic produce luxuriant butyrous colonies surrounded by a yellow zone signifying fermentation of mannitol. Such colonies must be confirmed as pathogenic strains by the coagulase test (ability of a loopful of the colony to solidify or produce a precipitate in 1 mℓ of rabbit, human, or horse plasma at 35 to 37°C in 4 to 24 hr). Nonpathogenic cocci (e.g., *Staphylococcus epidermidis*) produce small to medium butyrous colonies without a yellow zone. It should be stressed that spore-forming members of the genus *Bacillus*, yeasts, and certain molds also grow on this medium. The bacilli generally produce a more mucilagenous or mucoid type of growth. Gram stains should be performed on all acidogenic colonies. If there is doubt as to whether the strains are fermentating or oxidizing the mannitol, plates can be seeded and then overlayed with 2 to 3 mℓ of plain agar. Media such as Baird-Parker agar or Vogel-Johnson agar are frequently used in place of Mannitol-Salt agar. Details can be obtained from the manufacturer.

Kligler Iron Agar Slant (KIA): pH 7.4 ±

	grams per liter
Peptone	20.0
Lactose	10.0
Glucose	1.0
Sodium chloride	5.0
Ferric ammonium citrate	0.5
Sodium thiosulfate	0.5
Agar	15.0
Phenol Red	0.025

KIA is an extremely useful medium for differentiating among members of the enterobacteriaceae on the basis of their ability to either ferment glucose alone or ferment glucose and lactose. Fermentation of glucose is shown by a yellow butt (with or without a separation of the agar in the butt due to gas formation) and a red or alkaline slant (e.g., *Salmonella*, *Shigella*, and *Proteus* or any other lactose-negative form). A yellow butt plus a yellow slant indicates fermentation of both glucose and lactose. Production of H_2S is shown by a blackening in any part of the medium resulting from precipitation of iron sulfide in the presence of the ferric salt indicator. It is important that the butt be of the proper depth and the slant of the proper length. Inoculate from the center of a single colony out of EMB, MacConkey's, etc. by streaking the slant and stabbing the butt so as to get aerobic and relatively anaerobic conditions. One notes that the medium contains ten times more lactose than glucose. Stoichiometric fermentation of glucose in the butt does not release sufficient monocarboxylic acids to the upper slant to effect a pH change in the phenol red indicator when all of the glucose is fermented. Lactose-positive organisms continue to ferment the second sugar and then produce an upward migration of acid from the butt to acidify the slant. Thus, coliform organisms (*E. coli*, *Citrobacter*, *Enterobacter*, *Klebsiella*, etc.) produce a yellow butt and yellow slant.

Readings should be made at 24 and 48 hr. Frequently an acid slant will revert to alkaline by oxidation of the acetate and pyruvate via the citric acid cycle. KIA is an exceedingly useful medium in the clinical lab and is an example of biochemical theory reduced to practice.

Triple Sugar Iron Agar Slant (TSI): pH 7.3 ±

TSI is supplemented KIA agar to which sucrose has been added to permit separation of lactose-negative *Proteus* strains from lactose-negative *Salmonella* strains that cannot be accomplished with the double sugar medium of Kliger.

	grams per liter
Peptone	20.0
Lactose	10.0
Sucrose	10.0
NaCl	5.0
Glucose	1.0
Ferrous ammonium sulfate	0.20
Sodium thiosulfate	0.20*
Agar	13.0
Phenol red	0.025

* Present at more than twice this concentration in KIA.

One notes the same ratio of disaccharides to glucose as in KIA to explain acidification of the slant and/or butt region (stoichiometric fermentation of sugars in fixed proportions and upper migration of acid). Since Fe_2S_3 is yellow and FeS is black, the presence of ferrous iron (TSI) instead of ferric (KIA) requires less reducing potential, and thus the $Na_2S_2O_3$ concentration is lower in TSI. Thus, a blackening precipitate (FeS) appears as an indicator of H_2S production (the H_2S is derived from amino acids in the peptone and not from reduction of inorganic $SO_4^{=}$). A summary of TSI reactions is shown in Table 6.

Lysine Iron Agar LIA Agar: pH 6.7 ±

LIA agar was developed for the detection of Arizona strains, especially those which ferment lactose rapidly. It is especially useful in the diagnosis of typhoid fever and dysentery and other febrile enteric diseases. *Salmonella* and Arizona cultures produce lysine decarboxylase and rapidly form H_2S in large amounts. Decarboxylation of lysine is seen by purple butt and slant (alkaline reactions).

	grams per liter
Peptone	5.0
Yeast extract	3.0
Glucose	1.0
L-Lysine	10.0
Ferric ammonium citrate	0.5
Sodium thiosulfate	0.04
Brom Cresol purple	0.02
Agar	13.50
H_2O	1 ℓ

LIA agar can detect fermentation of glucose, decarboxylation of lysine, and oxidative deamination of lysine, although it is not a substitute for the standard lysine decarboxylase test. One notes the 10:1 ratio of lysine to glucose. Only those organisms that fail to decarboxylate lysine will produce an acid (yellow) butt. Those that ferment the 1% glucose will continue with decarboxylation of lysine converting it from an amphoteric amino acid to a basic substance. The alkalinity of the diamine diffuses throughout to yield a purple color in both butt and slant. A summary of LIA reactions is shown in Table 7.

Table 6
SUMMARY OF TSI REACTIONS OF THE ENTEROBACTERIACEAE

Inoculate slant and stab butt from an isolated colony on EMB or MacConkey's agar or from TSA slant made from such an isolated colony

Organism	Slant	Butt	Gas	H_2S	CO test
Ewings Section I[a]					
Escherichia	A(K)	A	+ (−)	−	Negative
Shigella	K	A	−	−	Negative
Salmonella typhi	K	A	−	+ (−)	Negative
Other Salmonellae	K	A	+	+ + +(−)	Negative
Arizona	K(A)	A	+	+ + +	Negative
Ewing's Section II[b]					
Citrobacter	K(A)	A	+	+ + +	Negative
Edwardsiella	K	A	+	+ + +	Negative
Klebsiella	A	A	+ +	−	Negative
Enterobacter	A	A	+ +	−	Negative
E. hafniae	K	A	+	−	Negative
Serratia	K(A)	A	−	−	Negative
Proteus vulgaris	A(K)	A	+	+ + +	Negative
P. mirabilis	K(A)	A	+	+ + +	Negative
P. morganii	K	A	− (+)	−	Negative
P. rettgeri	K	A	−	−	Negative
Providencia	K	A	+(−)	−	Negative

Note: Cultures resembling *Salmonella* can be tested directly with polyvalent O antiserum (one drop of heavy saline suspension from the slant plus one drop of antiserum on a microscopic slide; agglutination can be seen within a few minutes grossly). Further typing can be then rendered with specific group O antiserum. Details on the serological tests performed as slide or tube agglutination with somatic (O) or flagella (H) antisera are provided in the selected references. ( ), occasional reaction; K, alkaline; A, acid.

[a] Produce epidemic and endemic enteric disease.

[b] Occasionally enteric pathogens which also may produce serious infections in extraintestinal sites.

Simmon's Citrate Agar and the IMVIC test

Simmon's Citrate Agar: pH 6.9 ±	grams per liter
Ammonium dihydrogen phosphate	1.0
Dipotassium phosphate	1.0
Sodium chloride	5.0
Sodium citrate	2.0
Magnesium sulfate	0.2
Agar	15.0
Bromthymol blue	0.08

Simmon's citrate agar is the medium of choice for performing the citrate test, one of the four components of the classical IMVIC series of reactions (an acronym derived from Indole, Methyl red, Voges-Proskauer, and Inorganic Citrate).

Table 7
SUMMARY OF LIA REACTIONS OF ENTEROBACTERIACEAE

This medium is to be used in connection with TSI especially for Salmonella detection

LD conventional test[a]	Organism	Slant	Butt[b]	Gas	H_2S
d	*Escherichia*	K (purple)	K, Nor A	− or +	−
−[a]	*Shigella*	K	A (yellow)	−	−
+	*Salmonella*	K	K or N	−	+ (−)
+	*S. typhi*	K	K	−	+ or −
+	*Paratyphi A*	K	A (yellow)	+ or −	− or +
+	*Arizona*	K	K or N	−	+ (−)
−[a]	*Citrobacter*	K	A (yellow)	− or +	+ or −
+	*Edwardsiella*	K	K	− or +	+
+	*Klebsiella*	K or N	K or N	+ or −	−
	Enterobacter	K or N	A (yellow)	+ or −	−
	cloacae				
+	*aerogenes*	K	K or N	+ or −	−
+	*hafniae*	K	K or N	− or +	−
+	*Serratia*	K or N	K or N	−	−
	Proteus				
−[a]	*vulgaris*	R[c]	A (yellow)	−	− (+)
−[a]	*mirabilis*	R[c]	A (yellow)	−	− (+)
−[a]	*morganii*[d]	K or R[c]	A (yellow)	−	−
−[a]	*rettgeri*	R[c]	A (yellow)	−	−
−[a]	*Providencia*	R[c]	A (yellow)	−	−

Note: ( ), occasional reaction; K, alkaline; A, acid; N, no change; d, different biochemical types.

[a] Note the correlation between a failure to decarboxylate lysine (without subsequent production of basic diamine) and acidification of the butt due to glucose fermentation (N slant, A butt).

[b] Theoretically all should be acid by definition of the Enterobacteriaceae, but lysine decarboxylase alkalinity masks the acid produced by glucose.

[c] R, red color due to deamination of lysine.

[d] *Morganella morganii.*

The production of indole from tryptophane is detected by the addition of Kovac's reagent (*p*-dimethyl amino benzaledehyde dissolved in amyl alcohol and concentrated HCl) to peptone broth with the rapid visualization of a cherry red color in the amyl alcohol layer.

The methyl red test is an assay for acid production from glucose in a buffered broth. A few drops of an alcoholic solution of methyl red is added to a 48-hr culture and an immediate red color indicates a positive reaction (extensive glycolysis). Negative tests produce a yellow color. Equivocal red-orange colors should be repeated with tests incubated for 4 to 5 days.

The Voges-Proskauer test is performed on an aliquot of the same tube of buffered peptone glucose broth as was used for the methyl red test (MR-VP broth). To 1 mℓ of MR-VP broth add the VP reagent.* A positive test is indicated by the development of a pink color with the final reading taken at 4 hr. This test is based upon the formation of acetylmethyl-carbinol from glucose, which in turn is oxidized in an alkaline medium to diacetyl. Diacetyl reacts with the creatine present in peptone to form a pink color.

The citrate test refers to the ability of an organism to utilize sodium citrate as a sole source of carbon. Those organisms capable of assimilating Na citrate will grow on the surface of the agar slant or in the broth and produce an alkaline pH (blue color). Most bacteria, of course, can utilize citrate as a sole source of carbon including *E. coli* but the utilization is pH dependent. At low pH tricarboxylic acids such as citrate are not ionized and pass freely

* 15 drops 5% alpha-naphthol in absolute EtOH followed by 10 drops of 40% KOH.

Table 8
TYPICAL IMVIC REACTIONS

	I	M	V	IC
E. coli	d[a]	+	−	−
Enterobacter	−	−	+	+
Klebsiella	−	−	+	+
Salmonella	−	+	−	d
Arizona	−	+	−	+
Citrobacter freundii	−	+	−	+
Serratia marcescens	−	−	+	+
Shigella	−	+	−	−
Proteus vulgaris	+	+	−	d
Proteus mirabilis	−	+	−	+
Providencia	+	+	−	+

[a] d, different biochemical types.

through the cell walls and cytoplasmic membrane to be metabolized via the Krebs cycle. The citrate test involves a special set of conditions buffered at pH 6.9 to 7.0 so as to discriminate between those organisms which are less susceptible to the steric hinderance associated with the transport of anions above the isoelectric point of the cell. A summary of IMVIC reactions is shown in Table 8.

Sabouraud Dextrose Agar: pH 5.6 ±

	grams per liter
Dextrose	40.0
Polypeptone peptone	10.0
Antibiotics	As required
Agar	15.0

Sabouraud dextrose agar may be used for the isolation, identification, and maintenance of pathogenic and nonpathogenic fungi. The low pH tends to partially repress growth of bacteria. Most fungi grow as well at pH 7 as at pH 5 to 6. Sabouraud dextrose agar can be prepared at pH 6.9 to 7.2, but antibiotics must be added to make it selective. Isolation of pathogenic fungi from heavily contaminated clinical material can be obtained by addition of penicillin G (20 units/mℓ) and streptomycin (40 μg/mℓ) immediately before use. For a more stable medium, chloramphenicol alone is added (0.05 g/ℓ) or tetracycline at 100 μg/mℓ. If one is interested in isolation of pathogenic fungi only, the antibiotics cycloheximide (0.40 g/ℓ) and chloramphenicol (0.05 g/ℓ) are included (Mycosel Agar). Cycloheximide inhibits the common nonpathogenic strains of *Penicillium, Aspergillus, Trichoderma, Alternaria, Neurospora,* and other strains associated with clinical samples, but dermatophytes of the genera *Trichophyton, Microsporum,* and *Epidermophyton* are insensitive to this antibiotic. A quick screen for dermatophytes can be obtained by the use of DTM agar, which selectively inhibits bacteria and nonpathogenic fungi; dermatophytes produce a deep red color in the agar by alkaline reaction.

Casein-Soybean Digest Broth (Trypticase Soy Broth): pH 7.3

	grams per liter
Peptone (tryptic digest of USP casein)	17.0

Papaic digest of soy meal USP (phytone peptone)	3.0
NaCl	5.0
Dipotassium phosphate	2.5
Glucose	2.5
Distilled water	1 ℓ

This is an excellent all-purpose medium for the growth of fastidious organisms. Swabs from material with scanty exudates (e.g., some eye cultures) can be inoculated directly into the broth and then subcultured to selective media for speciation. Quantitative enumeration of bacteria in urine can also be determined by planting decimal portions (1.0 mℓ of undiluted urine to 1.0 mℓ of urine diluted 10^{-5}) for detection of bacteriuria. Casein-soybean digest broth is also useful for sterility tests in a quality control program designed to assay the sterility of parenterals, broth media, sterile reagents (saline, blood, transport media, etc.) autoclaved hospital supplies, and sterile devices. It is one of the media designated for the sterility testing of drugs and devices in the U.S. Pharmacoepia. A laminar flow-hood should be employed for all sterility tests, but is otherwise unnecessary.

Casein-Soybean Digest Agar (Trypticase Soy Agar): pH 7.3

	grams per liter
Peptone (tryptic digest of USP casein)	15.0
Papaic digest of soy meal (phytone peptone)	5.0
NaCl	5.0
Agar	15.0
Distilled water	1 ℓ

Casein-soybean digest agar will support growth of the same range of organisms as the corresponding broth. It can be used as a base for blood agar or chocolate agar. With or without blood, it is a useful all-purpose agar medium to be used wherever total aerobic plate counts are desired (e.g., urine cultures) either by the pour plate method or by surface streaking of calibrated loops to tempered plates. Casein-soybean digest agar plates with or without blood are also useful as settling plates to determine the number of bacteria in the air either while working at a laminar flow hood (e.g., when doing tissue culture work or performing an aseptic fill) or for general monitoring of the microbial environment for contamination control in the lab, ward, or operating room. Addition of glucose must be considered if it is to be considered nutritionally similar to TSB.

Carbohydrate Fermentation Media

Even though rapid diagnostic kits are becoming increasingly popular to determine carbohydrate fermentation, a variety of older basal media are still available to which sterile solutions of filtered carbohydrates are added with or without addition of an inverted test tube (Durham) to detect gas (e.g., phenol red broth which contains peptone, beef extract, NaCl, and the appropriate carbohydrate). Lactose fermentation is frequently used for detection of coliform organisms in water and dairy products as well as for the identification of fecal coliforms and *E. coli*, in which case gas is produced from lactose in the presence of boric acid at 42°C. The above type of medium can be employed without a pH indicator since gas production is the criterion measured. Lactose is a relatively heat stable carbohydrate and can be added directly to the broth before autoclaving. Production of gas automatically indicates acid production since the H_2 and CO_2 produced are derived from dissimilation of the pyruvate and lactate.

One of the most effective means of determining carbohydrate fermentation is to inoculate Cystine Trypticase agar (CTA) stabs. To the CTA semisolid medium weighed out Q.S. for 1 ℓ, add a sterile filtered stock solution to give a 0.5 to 1.0% final concentration of the desired carbohydrate, bring to a boil for 1 min, dispense in screw cap tubes (1/2 full), autoclave and store at room temperature for use (*Note:* without added carbohydrate CTA is an excellent maintenance media and can be used to detect motility). Fermentation can be noted by yellowing of a deep stab if the sugar has been incorporated into the CTA.

Employing CTA base without added sugar, fermentation can be noted by placing a commercially available filter paper carbohydrate disc into the tube and then stabbing the disc at the same time into the medium. Acid and gas can be seen in the area of the disc; the bottom of the tube serves as a carbohydrate-free control. Fermentation can also be detected by employing tubes of phenol red broth base (disc immersed) or phenol red agar pour plates (steak surface with organisms; place the carbohydrate disc on the surface, press firmly, and incubate-yellow zone around the disc indicates fermentation). However, only use of the stab culture or the Durham tube (inverted vial) can reveal gas production

CTA Agar: pH 7.3 ±

	grams per liter
Cystine	0.50
Trypticase peptone	20.0
Agar	2.5
NaCl	5.0
Sodium sulfite	0.5
Phenol red	0.017
Water	1 ℓ

FTM (Fluid Thioglycollate Medium): pH 7.1 ±

About 0.1 g of $CaCO_3$ should be placed into each tube if lactic or propionic acid bacteria or members of the genus *Clostridium* are to be maintained in this medium.

	grams per liter
Pancreatic digest of Casein (USP)	15.0
L-Cystine	0.5
Dextrose	5.0
Yeast extract	5.0
Sodium chloride	2.5
Sodium thioglycollate	0.5
Resazurin (redox indicator)	0.001
Agar	0.750

Note: Store at room temperature in screw cap tubes; boil up fresh before use if more than the upper 1/3 of the tube is oxidized. Do not boil again if broth becomes reoxidized.

FTM will support the growth of fastidious organisms such as streptococci and lactobacilli without addition of ascitic fluids. Swabs can be inoculated directly into tubes of FTM and subcultures made to selective media. The growth of organisms in the lower reduced zone indicates the possible presence of an obligate anaerobe. Facultative organisms or aerobes will grow throughout the medium and in the upper oxidized zone (pink resazurin layer). Direct inoculation of a specimen is recommended whenever the clinical picture or Gram

stain suggests an obligate anaerobe. This broth should be used in conjunction with TSB for sterility work on parenterals, broth media, and sterile reagents (see USP 20). The presence of sulfhydryl groups not only lowers the redox potential but also inactivates mercurials. Thus, FTM was originally designed for sterility tests for formulations containing Hg preservatives and not for the propagation of anaerobes. FTM is useful in isolating organisms from blood. The pathogenic protozoan *Trichomonas vaginalis* can be grown in this medium if the pH is adjusted to 6.0 and human, calf, or horse serum is added to a final concentration of 5.0% v/v.

Cooked Meat Medium pH 7.2 ±

	grams per liter
Dextrose	2.0
Polypeptone peptone	20.0
Beef heart	454
Sodium chloride	5.0

Cooked meat medium has been used for over 30 years for the propagation of obligate anaerobes. These organisms are able to sequester themselves in sulfhydryl-rich crevices within the meat surface. The low redox potential is maintained if the meat is cooked up fresh prior to use. In food laboratories cooked meat medium is useful for propagation of *Clostridium botulinum*. In the hospital laboratory it is useful whenever an anaerobe is suspected (wounds, genital tract, blood, etc.). Aerobic organisms grow equally well but frequently do not digest the meat to the same extent as members of the genus *Clostridium*. Any growth must be confirmed as an anaerobe by Gram stain and by demonstration that a colony picked from an anaerobic jar does not grow aerobically. Thus, cooked meat tubes must be subcultured to blood agar aerobically and anaerobically; subculture to FTM and TSB tubes is also useful in deciding if an obligate anaerobe is present, but only if one is dealing with a pure culture.

Hugh and Leifson OF Medium (Oxidation Fermentation Media)

If a Gram-negative rod produces no change on a TSI slant, it is considered to be a nonfermenter. One must next determine if it is capable of oxidizing carbohydrates. OF medium is aseptically dispensed into tubes as 5 mℓ semisolid stabs. It is useful in the study of nonfermenting Gram-negative rods which must be bifurcated into oxidizers or nonoxidizers. The culture is stabbed to the bottom into duplicate tubes for each sugar (add the desired carbohydrate solution to 1.0% final concentration prior to sterilization; autoclave only 10 min). Cover one tube of each pair with 5 mm of sterile mineral oil and incubate for 48 hr or longer. Record presence of acid (yellowing of medium) in both tubes. Fermentative organisms will produce color changes in both the open and closed tubes. Oxidative organisms will produce yellowing of the open tube at the surface but no change in the closed tube (even though growth in the closed tube may occur). The low concentration of agar in this semisolid medium also allows one to note motility of the organisms by migration from the vertical stab line (similar to CTA stab). Hugh and Leifson OF medium takes advantage of our knowledge of glycolysis and the direct oxidation of hexoses for those organisms not possessing an Embden-Meyerhoff system. It is important to include the closed control, lest rapid and extensive oxidation be confused with true fermentation. A more complete appreciation of the value of the OF system can be obtained from the following differential chart, where one notes its value in identifying *Pseudomonas*, *Acinetobacter*, *Alcaligenes*, *Achromobacter*, and *Flavobacterium* (Table 9). Included in Table 9 are data on the cytochrome oxidase test and growth on SS agar so as to give a more complete synopsis of the tests for

Table 9
OF MEDIUM GLUCOSE OXIDATION BY NONFERMENTERS AND OTHER PERTINENT REACTIONS

	CO	Glucose open	Glucose sealed	Motility	SS agar
Pseudomonas	+	A	0	+	+
Alcaligenes	+	0	0	+	0
Moraxella	+	0	0	0	0
Acinetobacter	0	A, 0	0	0	0
Achromobacter	+	A	0	+	+
Flavobacterium	+	A	0 (+)	0 (+)	0
Fermenter control	0	A	A		

Note: +, positive; A, acid; 0, no change; (+), delayed or occasional.

nonfermenting bacilli (NFB). Miniaturized rapid diagnostic kits have also been developed for this group of organisms (e.g., API).

Candle Jar

Carbon dioxide behaves as a growth supplement for many bacteria. The simplest way of producing an atmosphere of carbon dioxide is to light a candle inside a large wide-mouthed jar. The petri dishes are placed within the jar, the candle is lit, and the lid screwed in place. Within about 1 min the candle will extinguish due to the blanketing effect of CO_2, which is heavier than air. Accordingly, one has produced an atmosphere of approximately 10% CO_2; the microaerophilic environment encourages growth for some species by the combined removal of oxygen and the addition of CO_2. The biochemical basis for CO_2 enrichment is not clearly understood, but it is known that CO_2 fixation occurs regularly in certain metabolic pathways. The candle jar enhances the growth of Gram-positive and Gram-negative cocci as well as strains of *Hemophilus*.

ANAEROBIC ORGANISMS

Anaerobes in pure or mixed cultures are present in over 80% of the clinical abscesses associated with human or veterinary tissues. Successful isolations can be accomplished in most (but not all) cases with the use of commercially available anaerobe jars, such as the BBL Gas-Pak® system, which is available from most bacteriological supply houses. In this system, water is added to an envelope which then generates a mixture of H_2 and CO_2 into a sealed chamber. The O_2 passes through a dry catalyst and "cold combustion" occurs. A strip of filter paper, impregnated with the methylene blue, is included in the kit as an anaerobic indicator. It will slowly change from blue (oxidized methylene blue) to colorless (reduced methylene blue). For best results, pour plates of blood agar from tubes of prereduced agar are used, or a medium that has been freshly rehydrated and autoclaved the day of use. Some obligate anaerobes will not grow in the Gas-Pak® if prereduced media is not employed.

Prereduced media generally contain L-cysteine (0.05%) and are made under a stream of CO_2 in the absence of oxygen. When made in this manner and tightly sealed, an anaerobic environment is maintained. Every time the culture is opened for examination or transfer a CO_2 gas "flush" is required to wash out oxygen. Details on the preparation of a variety of prereduced media have been published.[9,10]

Specimens to be examined for anaerobes include (1) abscessess or drainage from any part of the body, (2) postsurgical or other infections, (3) wounds, (4) burn infections, and (5) blood. Little is to be gained in taking routine anaerobic cultures of throat swabs, urine, or

fecal samples because obligate anaerobes are normal to these sites. Oral, bronchial, and brain abscesses should also be checked for *Actinomyces* (candle jar). Genital, oral, or bronchial infections should be observed with phase or dark filed microscopy for spirochetes. About 80% of all specimens from abscesses, wounds, or infected sites will contain obligate anaerobes if properly cultured in a prereduced system. About 65% of these specimens will also contain facultative bacteria which can be distinguished by (1) careful attention to colonial morphology, (2) Gram stain, and (3) regrowth after aerobic subculture. Anaerobes may not be isolated if the specimen is cultured in broth medium before direct streaking (broth enrichment); the more fastidious obligate anaerobes will be overgrown by less fastidious facultative bacteria. Abscesses and wounds can yield up to seven different kinds of anaerobes and for plural and unogenital infections up to four may be isolated.

The genera of obligately anaerobic bacteria are as follows:

Rods

Form spores	*Clostridium* Gram positive)	
Do not form spores	*Proprionibacterium*	motile and Gram positive
	Arachnia	
	Bifidobacterium	
	Lactobacillus	
	Actinomyces	
	Eubacterium	
	Fusobacterium	motile and Gram negative
	Leptotrichia	
	Bacteroides	
	Butyrivibro	
	Succinivibrio	
	Succinimonas	
	Vibrio	
	Selenomonas	
	Treponema	

Cocci

Gram positive	*Peptostreptococcus*
	Peptococcus
Gram negative	*Veillonella*
	Acidaminococcus

The following is a list of pathogenic obligate anaerobes isolated from clinical specimens:

* *P. acnes* (skin, intestinal tract)
* *P. avidum*
* *Lactobacillus catanaforme* (pleural)
* *Bifidobacterium eriksonii* (intestinal tract)
*** *Eubacterium lentum* (frequently in abscesses)
*** *E. limosum* (frequently in infections)
** *E. alactolyticum* (pleural)
*** *Bacteroides fragilis* (blood, wounds, abscesses but rare in pleural specimens)
*** *B. vulgatus*
** *B. oralis* (infections of mouth and URI)
** *B. corrodens* (mouth)
** *Fusobacterium fusiforme* (mouth or UR abscesses)
** *F. mortiferm* (abscesses)
** *F. naviforme* (abscesses)

** *F. nucleatuim* (mouth, URI)
** *F. necrophorum*
** *F. russii* (abscesses)
** *F. varuim* (abscesses)

Note: * infrequent, ** occasionally, *** frequent, URI, upper respiratory infection.

Treponema

The incidence of many types of treponemes in clinical material is poorly established (except for positive dark-filed examination of a syphilitic lesion). *T. vincentii* is isolated from the oral cavity and *T. refringens* (formerly called *Borrelia refringens* or *T. genitalis*) is isolated from oral or genital material.

Clostridium

Clostridium perfringens usually makes up 50% of the clostridia isolated from clinical material. This organism sporulates poorly. Other clostridia can be shown to be sporogenous by Gram stain after 3 to 7 days on a slant of cooked media agar at 30°C. Clinical specimens will frequently harbor a group of nonpathogenic clostridia as follows: *C. inocuum*, *C. sordelli*, *C. bifermentans*, *C. sporogenes*, *C. septicuum*, *C. ramosum*, *C. sphenoides*. *C. sporogenes* is regarded as a nontoxigenic variant of *C. botulinum*.

The pathogenic clostridia, as judged by toxic production in culture (mouse i.p. assay) or pathogenicity in guinea pigs (i.m.) are as follows (t = produces toxin, n = necrosis): *C. histolyticum* (n), *C. limosum* (n), *C. perfringens* (t), *C. septicum* (t), *C. chauvoei* (t), *C. botulinum* (t), *C. carnis* (n), *C. novyi* (t), *C. haemolyticum* (t), *C. sordelli* (t), *C. difficule* (n).

The isolation and identification of obligate anaerobes requires special skill and patience. Unlike aerobic Gram-negative rods, a simple battery of biochemical tests is inadequate. When working with these organisms the bacteriologist should pay great attention to the source of the organisms, the Gram stain, and colonial morphology. As with all clinical isolates, the performance of appropriate antibiotic sensitivity tests is invaluable.

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