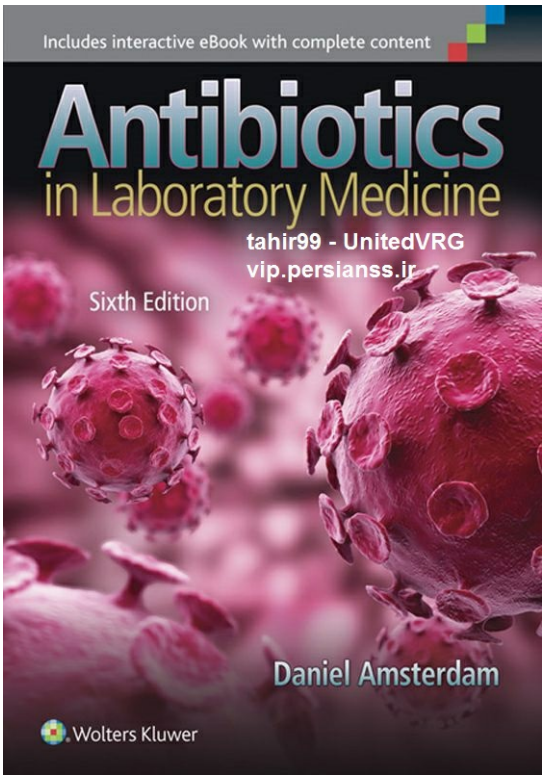
Includes interactive eBook with complete content
Antibiotics
in Laboratory Medicine
tahir99 - UnitedVRG
vip.persianss.ir
Sixth Edition
Daniel Amsterdam
Wolters Kluwer

Antibiotics in Laboratory Medicine

Sixth Edition

Antibiotics in Laboratory Medicine

Sixth Edition

Daniel Amsterdam, PhD, ABMM, FAAAS, FIDSA
Professor
Departments of Microbiology & Immunology, Medicine, and Pathology
School of Medicine and Biomedical Sciences
State University of New York at Buffalo;
Chief of Service, Department of Laboratory Medicine
Erie County Medical Center
Buffalo, New York

Wolters Kluwer
Philadelphia • Baltimore • New York • London
Buenos Aires • Hong Kong • Sydney • Tokyo

Acquisitions Editor: Julie Goolsby
Product Development Editors: Kristina Oberle and Andrea Vosburgh
Production Project Manager: Joan Sinclair
Design Coordinator: Terry Mallon
Manufacturing Coordinator: Beth Welsh
Prepress Vendor: Absolute Service, Inc.

6th edition

9 8 7 6 5 4 3 2 1

Printed in China.

Library of Congress Cataloging-in-Publication Data

Antibiotics in laboratory medicine / [edited by] Daniel Amsterdam. — Sixth edition.
p. ; cm.
Includes bibliographical references and index.
ISBN 978-1-4511-7675-9 (alk. paper)
I. Amsterdam, Daniel, editor.
[DNLM: 1. Microbial Sensitivity Tests. 2. Anti-Bacterial Agents—pharmacology. 3. Drug Resistance, Microbial—physiology. QW 25.5.M6]
QR69.A57
615.3’29—dc23

2014022704

For Victor Lorian, MD (deceased), my mentors and coauthors in this and other works, and as always, for my steadfast wife, Carol, and my children, Jonathan and Valerie.

Acknowledgments

As a former contributor to *Antibiotics in Laboratory Medicine*, I am pleased and honored to take on the editorial leadership of the sixth edition to carry on the vision of the founding editor, Victor Lorian, MD. Dr. Lorian's editorial direction spanned more than 30 years and this acknowledgment is a small tribute to his role. In the nascent edition, there were only antibacterial agents deliberated. Succeeding editions witnessed the expanding role of antifungal and antiviral compounds.

In addition to Dr. Lorian's vital contribution, this edition acknowledges the efforts of chapter authors from previous editions who were unable to continue in this work.

Preface

Since their discovery and introduction into the armamentarium of medicines, antibiotics have saved countless lives and contributed to the rapid advancement of modern medicine. The legacy drugs, penicillin, tetracycline, and their contemporary successors have been essential in sustaining health and dealing with human diseases. Their use to treat infections should be considered a global health resource that needs to be carefully conserved. Yet, we are confronted by the expanding scope of drug resistance and more pointedly multidrug resistance, which highlights the reliance on laboratory antimicrobial susceptibility and resistant gene testing. It has become patently clear that for many organisms once considered reliably susceptible to a number of broad-spectrum agents, unexpected resistance can occur as a result of foreign travel or multidrug courses of therapy during a hospital admission.

In recent editions of *Antibiotics in Laboratory Medicine*, which have now has spanned more than 35 years, nearly half the antibiotic era, readers have witnessed the role of newer compounds and drug classes including antifungal and antiviral agents, albeit limited the past decade, and had access to methods and approaches for determining efficacy and detailing the mechanisms of action and resistance of these compounds in vivo and in vitro.

New for this edition are the introductory "Perspectives" and the Appendix. The former deals with the outlook of antiinfective compounds; their origin and archeologic niche in nature; and their current role, development, and future application. In the Appendix are the Web addresses that readers can select for learning more about the nature of the drugs, current resistance patterns and trends, and their clinical application. Each of the intervening chapters, by new or former author(s) expanded and reworked, represent current science and laboratory practice. Several aspects of this volume are worth highlighting. In Chapter 1, Winkler et al. detail the criteria and considerations that Clinical and Laboratory Standards Institute uses in defining breakpoints, the essential divide between the classification of agents as "susceptible" or "resistant" or as we also know as "nonsusceptible." Examples and rationale are presented for changing breakpoints. In ensuing chapters, Drs. Turnidge and Bell and I expand on previously detailed information in experimentally defining susceptibility/resistance when using agar- or broth-based analytical systems. Drs. Venugopal and Hecht expertly update susceptibility of anaerobes, which, although essential, has recently become a less focused clinical challenge. Drs. Thompson and Patterson discuss and refine the testing modalities and clarified terms for defining and interpreting the activity of antifungal agents. Dr. Inderlied along with new coauthor Dr. Edward Desmond addresses the expanding role of the laboratory in evaluating the susceptibility of the mycobacteria to antimycobacterial agents. Dr. Rolain provides readers with a comprehensive review of susceptibility testing of unusual microorganisms for which there is a paucity of information. In Chapter 8, Dr. Edberg and new coauthors Drs. Latte and Sordillo update application and methods for measuring levels of antimicrobial agents in body fluids. Dr. Sundsfjord and colleagues from Norway in Chapter 9 examine the varied molecular methods that have come into use for detection of antibacterial resistance genes. In the ensuing Chapter 10, Dr. Stratton updates the molecular mechanism of action of antimicrobial agents, which is essential to understanding the MOA of drugs and designing new compounds. The chapter addressing antiviral agents (Chapter 11) discusses the several newly developed antiviral classes for HIV, cytomegalovirus, and influenza. Dr. Ostrov in collaboration with Dr. Amsterdam have reorganized and updated the chapter that addresses antiseptics and disinfectants. In Chapter 13, Drs. Frimodt-Møller and colleagues have done an extensive update on the evaluation of antimicrobial agents in experimental animal infections; new models and more than 50 new references have been added to the previously cited reference list. In the last work, Chapter 14, Dr. Bamberger and colleagues have used their vast clinical experience and knowledge and document the distribution of antimicrobial agents in extravascular compartments.

As a former (and current) contributor, I am cognizant of and in awe of my fellow authors who participated in this work and made this edition of *Antibiotics in Laboratory Medicine* scientifically sound and clinically meaningful. The sixth edition is authored by an international group of distinguished scientists and physicians, expert in their discipline, brilliant in their vision, and recognized worldwide.

Daniel Amsterdam

Paul G. Ambrose, PharmD
Associate Research Professor
Pharmacy Practice
School of Pharmacy and Pharmaceutical Services
University at Buffalo
Amherst, New York

Daniel Amsterdam, PhD, ABMM, FAAAS, FIDSA
Professor
Departments of Microbiology & Immunology, Medicine, and Pathology
School of Medicine and Biomedical Sciences
State University of New York at Buffalo;
Chief of Service, Department of Laboratory Medicine
Erie County Medical Center
Buffalo, New York

David R. Andes, MD
Professor
Departments of Medicine and Microbiology
Chief, Division of Infectious Diseases
University of Wisconsin
Madison, Wisconsin

David M. Bamberger, MD
Professor of Medicine
University of Missouri-Kansas City School of Medicine;
Chief, Section of Infectious Diseases
Truman Medical Centers
Kansas City, Missouri

Jan M. Bell, BSc (Hons), BA
SA Pathology
Women's and Children's Hospital
North Adelaide, Australia

Franklin R. Cockerill III, MD
Ann and Leo Markin Professor of Medicine and Microbiology
Department of Laboratory Medicine and Pathology
Mayo Clinic College of Medicine
Rochester, Minnesota

Edward Desmond, PhD, DABMM
Chief
Myocobacteriology and Mycology Section
Microbial Diseases Laboratory
California Department of Public Health
Richmond, California

Stephen C. Edberg, PhD, ABMM, FAAM
Professor Emeritus
Yale University;
Mount Sinai Health System
New York, New York

John W. Foxworth, PharmD
Professor of Medicine, Bioinformatics and Personalized Health
Department of Medicine, Division of Clinical Pharmacology
University of Missouri School of Medicine

Kansas City, Missouri

Niels Frimodt-Møller, Professor, MD, DMSc
Senior Consultant
Department of Clinical Microbiology
Hvidovre Hospital
Hvidovre, Denmark

Dale N. Gerding, MD
Professor
Department of Medicine
Loyola University Chicago Stritch School of Medicine
Maywood, Illinois

David W. Hecht, MD, MS, MBA
Senior Vice President of Clinical Affairs/Chief Medical Officer
Department of Medicine
Loyola University Medical Center
Maywood, Illinois
Department of Medicine
Hines VA Hospital
Hines, Illinois

Joachim Hegstad, MSc
Department of Microbiology and Infection Control
University Hospital of North Norway
Tromsø, Norway

Kristin Hegstad, PhD
Reference Centre for Detection of Antimicrobial Resistance
Department of Microbiology and Infection Control
University Hospital of North Norway;
Research Group for Host-Microbe Interactions
Department of Medical Biology
University of Tromsø The Arctic University of Norway
Tromsø, Norway

Clark B. Inderlied, PhD
Emeritus Professor of Clinical Pathology
University of Southern California
Keck School of Medicine
Los Angeles, California

Michael A. Kallenberger, PharmD
Clinical Lead Pharmacist
Antimicrobial Stewardship
Truman Medical Centers
Kansas City, Missouri

Shelly Latte, MD
Attending Physician
Division of Infectious Diseases
Division of Hospital Medicine
St. Luke's and Roosevelt Hospital Center
Mount Sinai Health System
New York, New York

Alexander J. Lepak, MD
Assistant Professor
Department of Medicine
University of Wisconsin School of Medicine and Public Health
Madison, Wisconsin

Barbara E. Ostrov, MD

Vice Chair, Department of Pediatrics
Professor of Pediatrics and Medicine
Penn State Hershey Children's Hospital
Penn State Hershey Medical Center
Hershey, Pennsylvania

Thomas F. Patterson, MD, FACP
Professor of Medicine
The University of Texas Health Science Center at San Antonio;
Department of Medicine/Infectious Diseases
South Texas Veterans Health Care System
San Antonio, Texas

Brian S. Pepito, MD
Clinical Assistant Professor
Department of Medicine
University of South Dakota School of Medicine
Sioux Falls, South Dakota

Jean-Marc Rolain, PharmD, PhD
Professor
Faculté de Médecine et de Pharmacie
URMITE CNRS-IRD-INSERM
IHU Méditerranée Infection
Marseille, France

Ørjan Samuelsen, PhD
Reference Centre for Detection of Antimicrobial Resistance
Department of Microbiology and Infection Control
University Hospital of North Norway
Tromsø, Norway

Emilia Mia Sordillo, MD, PhD, FACP
Senior Attending
Departments of Medicine and Pathology and Laboratory Medicine
Medical Director, Microbiology and Molecular Diagnostics
Mount Sinai St. Luke's and Mount Sinai Roosevelt
Mount Sinai Health System
New York, New York

Charles William Stratton, MD
Director, Clinical Microbiology Laboratory
Vanderbilt University Medical Center
Nashville, Tennessee

Arnfinn Sundsfjord, MD, PhD
Professor
National Reference Laboratory for Detection of Antimicrobial Resistance
Department of Clinical Microbiology and Infection Control
University Hospital of North Norway;
Department of Medical Biology
Faculty of Health Sciences
University of Tromsø The Arctic University of Norway
Tromsø, Norway

George R. Thompson III, MD
Assistant Professor of Medicine
Department of Medical Microbiology and Immunology
Department of Medicine, Division of Infectious Diseases
University of California Davis
Davis, California

John D. Turnidge, MBBS, FRACP, FRCPA, MASM
Professor

Departments of Pediatrics, Pathology, and Molecular and Biomedical Sciences
University of Adelaide
Adelaide, South Australia

Anilrudh A. Venugopal, MD
Associate Professor
Department of Medicine
Loyola University Medical Center
Maywood, Illinois

Matthew A. Wikler, MD
Vice President
The Medicines Company
Parsippany, New Jersey

Contents

Perspectives

Intersection of Drug Development, Challenges of Antimicrobial Resistance, and Predicting Antimicrobial Efficacy

Daniel Amsterdam and Charles William Stratton

In 2009, the World Health Organization (WHO) referred to the problem of antibiotics and antibiotic resistance, stating, "Antibiotic Resistance – one of the three greatest threats to human health." In the 8 years since the last publication of this volume, there have been numerous and significant advances in our understanding of the effects of antimicrobial agents on the human (and animal) microbiome, the increase and recognition of new antimicrobial (i.e., antiinfective resistance mechanisms), as well as the tremendous advances in technology that have led to the detection of these multiloci mechanisms especially among the gram-negative Enterobacteriaceae. Unfortunately, progress in all these areas is dimmed by the apparent disinterest of pharmaceutical companies in the development of new, more effective compounds to combat the increasing number of drug-resistant infections. Several themes are incorporated into this perspective and limn the changing landscape of antimicrobial development and the means with associated technology for estimating efficacy in human or animal hosts. These are the constriction of the antibiotic development pipeline, origins of antibiotic resistance, developing knowledge about the human and animal microbiomes, the predictive value of antimicrobial susceptibility testing, and new technologies especially "next-generation" sequencing, which has the capability of examining the entire microbial sequence rapidly and for reasonable costs.

THE CONSTRICTED ANTIBIOTIC PIPELINE

In the United States and around the world, the incidence of drug-resistant infections and associated morbidity have increased. WHO identified the resistance of microorganisms to antimicrobial agents as one of the three greatest threats to human health. Recent reports by the Infectious Diseases Society of America (IDSA) (1) and the European Centre for Disease Prevention and Control and the European Medicines Agency (2) document that the number of candidate drugs in the developing pipeline, which are beneficial compared to existing drugs that will be capable to treat infections due to the group of pathogens termed "ESKAPE" (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) are few. The aforementioned six species/groups cause the majority of US hospital infections and are not always contained by the available armamentarium of antibacterial drugs (3).

It is the IDSA's view that the antibiotic pipeline problem can be dealt with by engaging global, political, scientific, industry, economic, intellectual property, policy, medical, and philanthropic leaders to develop creative incentives that will serve to stimulate new and ongoing research and development in this area. In this regard, it has been inferred that the financial gains and advantages for major pharmaceuticals may not be particularly advantageous for the development of new antimicrobial agents because the costs for treatment regimens, and reimbursement schedules in comparison to antineoplastic, biologic respiratory, and allergy drugs. In short, the economic advantage of antimicrobial agents relative to other drug class candidates presents an economic disadvantage (4).

Despite the confluence of these negative factors, it is the IDSA's aim that the "creation of sustainable global antibacterial drug R&D enterprise" achieve in the short term 10 new, safe, and effective antibiotics by 2020. Toward this end, IDSA (5) launched a new collaboration entitled the "IDX '20" initiative, which several American and European groups and societies have endorsed. This declaration is a noble effort that no doubt will be reviewed in the next few years—or by the next publication of *Antibiotics in Laboratory Medicine*.

The development of new antimicrobial agents is one of three strategies that have been proposed to meet the challenge of multiresistant diverse microorganism types (extended-spectrum β-lactamases [ESBLs], *Klebsiella pneumoniae* carbapenemase [KPC], methicillin-resistant *Staphylococcus aureus* [MRSA], vancomycin-resistant enterococci [VRE], etc.) collectively referred to as multidrug-resistant organisms (MDROs). The other two strategies include interrupting the cross-transmission of MDROs and effective pharmacology oversight-stewardship in the treatment of these infections. This latter strategy incorporates tactics for appropriate initiation, selection, and de-escalation of antimicrobial therapy.

TRACING THE ORIGINS OF ANTIBIOTIC RESISTANCE

The marvel of antibiotic discovery, now more than 70 years old, gave rise to an era of drug innovation and discoveries that have been tempered by the emergence of resistant microorganisms (6).

Almost every chapter in this volume addresses the detection and identification of microbes that are resistant to a particular drug or class of antiinfectives. In examining the history and development of antimicrobial resistance, should this be interpreted to mean that antibiotic resistance in clinically significant bacteria is a contemporary phenomenon? Recent studies of modern human (and environmental) commensal microbial genomes suggest that these genomes possess a greater concentration of antibiotic resistance genes than had been previously recognized (7–9). A highly varied collection of genes encoding resistance to β-lactam, tetracycline, and glycopeptide antibiotics was recently found in 30,000-year-old

Beningian permafrost sediments in Alaska (7). D'Costa and colleagues (7) documented through structure and function studies the complete vancomycin resistance element vanA and confirmed its similarity to modern variants. In earlier work, D'Costa et al. (10) analyzed the antibiotic resistance potential of soil microorganisms. In this study, it was alarming to discover that the frequency of high-level resistance detected in this study was to antibiotics that have served as the standard therapeutic regimens for decades. No class of antibiotic natural or synthetic was spared with respect to bacterial target. A summary of the 18 antibiotics and the extent of inactivation of the 480 strains that formed the library is noted in Figure 1. In general, without exception, investigators found that every strain in the library was resistant on average to 7 or 8 antimicrobials; two strains were resistant to 15 of the 21 drugs. Several antimicrobials, including cephalexin, the synthetic dihydrofolate reductase inhibitor trimethoprim, and the more recently developed lipopeptide daptomycin were almost universally ineffective against the library of bacterial strains. This wide dissemination of antibiotic resistance elements tempers the contemporary hypothesis for the emergence of antibiotic resistance and instead implies a natural history of resistance.

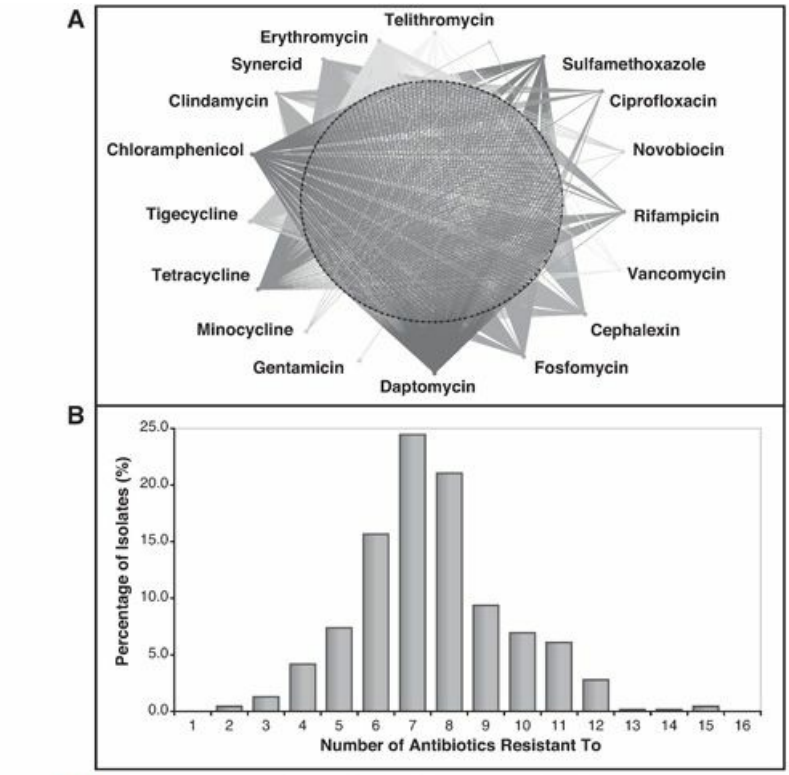

Figure 1 ■ Antibiotic resistance profiling of 480 soil-derived bacterial isolates. A: Schematic diagram illustrating the phenotypic density and diversity of resistance profiles. The central circle of 191 black dots represents different resistance profiles, where a line connecting the profile to the antibiotic indicates resistance. **B:** Resistance spectrum of soil isolates. Strains were individually screened from spores on solid *Streptomyces* isolation media (SIM) against 21 antibiotics at 20 mg of antibiotic per milliliter of medium (mg/mL). Resistance was defined as reproducible growth in the presence of antibiotic. (From D'Costa VM, McGrann KM, Hughes DW, et al. Sampling the antibiotic resistome. *Science* 2006;311:375.) (See Color Plate in the front of the book.)

PREDICTIVE VALUE OF ANTIMICROBIAL SUSCEPTIBILITY TESTING RESULTS

This volume is dedicated to tests that estimate the interactive end point of "bug" and drug. It is noteworthy, and without alarming revelation, that diseases other than those caused by infectious agents are treated by medicating the host. In contrast, therapy for infectious disease attempts to eliminate the pathogens from the host while minimizing adverse sequelae due to host immune responses and drug side effects. The target then for drug therapy is the pathogen; however, this frequently results in collateral damage.

A wide variety of manual and automated tests are described in this volume and in particular in Chapters 2 and 3, they review the development and detail of these assays during the latter half of the last century. Results of those assays are interpreted in the form of categorical values ("S," "I," or "R") or with numerical equivalents and defined by at least two major consensus groups—Clinical and Laboratory Standards Institute (CLSI) in the United States and European Committee on Antimicrobial Susceptibility Testing (EUCAST) in the European Union. In Chapter 1, Wikler and colleagues explore and discuss the reasoning/rationale for defining the "breakpoint," the dividing quantitative line between susceptibility and resistance that is the underpinning of antibacterial susceptibility tests. The question posed here is how successful are these results in predicting a positive outcome in patients with infection.

When using mortality as an outcome indicator, several studies (11–15) executed between 1996 and 2003 demonstrated that mortality was reduced by 40% to 60% when the first antimicrobial agent administered was "susceptible." Resistance as determined by in vitro testing is considered in these studies to be an independent risk factor for therapeutic failure. The advent of molecular methods to detect specific resistance genes augmented by whole genome sequencing (WGS) will no doubt enhance this capability (see the following section on "Impact of New Technologies" and Chapter 9 by Hegstad et al.). Clearly, the standard, that is, phenotypic antimicrobial susceptibility tests that we use today fail to mimic the physiologic status of the host in several dimensions. First, in the "test tube," the drug is in constant association with the host—not varying according to its pharmacokinetic construct; second, the host's cellular and antibody entities are absent; and last, the bioburden, that is, the test system agent concentration may be at variance from the true infectious dose extant in various body compartments.

When evaluating the expected correlation between results of in vitro susceptibility tests and therapeutic response, Rex and Pfaller (16) coined the "90–60 rule," which indicated that a susceptible result is associated with a favorable therapeutic response in 90% to 95% of patients. The

formulative predictions are beclouded when one considers immunocompromised patients with polymicrobic infections.

Several pertinent and directed questions can be posed to form the essential meaning of this section. Can antimicrobial susceptibility test results as performed in the routine clinical microbiology laboratory be translated into clinical efficacy and potency? For patients with MRSA bacteremia, is there a difference in patient outcome when reported minimum inhibitory concentrations (MICs) are in the susceptible category as defined by consensus organizations? Should there be a difference in categorical interpretation (S, I, or R) for pneumococcal meningitis–associated and non–meningitis-associated disease? When documented nosocomial bacteremia is caused by *P. aeruginosa*, is patient outcome associated with reduced piperacillin-tazobactam MICs?

Clearly, the response in each of the previously cited cases is the critical establishment of the breakpoint concentration by regulatory oversight groups that define the chasm between susceptible and "resistant." In Chapter 1 of this volume, Wikler and coauthors define this parameter and the necessary evidence to establish it. Simply stated, each antimicrobial agent/drug pair is dependent on the pharmacokinetic (PK) and pharmacodynamic (PD) properties of the antiinfective compound and the associated clinical outcome. Specific parameters that are pertinent to evaluating the PK/PD are the area under the curve of C_{max} (peak) and above the resulted bug–drug MIC, and the time duration that C_{max} is greater than the MIC (Fig. 1). The pharmacodynamics of an antimicrobial is then the sum of the antimicrobial PK plus the MIC and the clinical outcome. Generally, the application of PK/PD parameters for drugs can be categorized as those drugs which are concentration-dependent; that is, higher concentrations are required in relation to MIC to kill pathogens (e.g., fluoroquinolones and aminoglycosides) and time-dependent (concentration independent) agents whose effectiveness is measured by duration of exposure above a recognized inhibitory concentration (the MIC_{90}) to determine killing. Examples of this latter group include cell wall–active agents such as β-lactam antibiotics (penicillins and cephalosporins) and vancomycin.

In 2004, Sakoulas and associates (17) redefined the interpretive concentration for successful (i.e., enhanced) outcome in the interpretation of vancomycin MIC for bloodstream isolates (BSIs) or MRSA. They determined that when the MIC was less than or equal to 0.5 μg/mL, successful therapy resulted in a 55.6% improved outcome as compared to 9.5% when MICs were 1 or 2 μg mL—still in the susceptible category.

In 2008, the CLSI revised the susceptible category interpretation for penicillin based on the clinical syndrome (meningitis vs. non-meningitis) and the route of administration of penicillin. Table 1 outlines the standard pre- ("former") and post-2008 ("new") breakpoint interpretations. Although there was one susceptibility category (≤0.06 μg/mL) in the former standard, the new standard defined three categories based on clinical syndrome and route of administration (18).

Table 1

Penicillin and *Streptococcus pneumoniae*: Comparison of Former (pre-2008) and New Penicillin Breakpoints (MIC)—Clinical and Laboratory Standards Institute, 2008

	Susceptibility Category MIC (μg/mL)		
Standard	**Susceptible**	**Intermediate**	**Resistant**
Former—all clinical syndromes and penicillin routes	≤0.06	0.12–1	≥2
New (by clinical syndrome and penicillin route)			
Meningitis, IV penicillin	≤0.06	—[a]	>0.12
Nonmeningitis, IV penicillin	≤2	4	≥8
Nonmeningitis, oral penicillin	≤0.06	0.12–1	≥2

[a]No intermediate category defined under new standard.
MIC, minimum inhibitory concentration; IV, intravenous.

In a 2008 study of *P. aeruginosa* bacteremia patients treated with piperacillin-tazobactam, Tam et al. (19) determined that when susceptibility to piperacillin-tazobactam was 32/64 μg/mL, there was a fourfold increase in 30-day mortality (85.7% vs. 22.2%) compared to isolates with susceptibility of 16 μg/mL.

In each of the previously mentioned examples, credence for the predictive value of an MIC and the associated susceptible or resistant categorical interpretation can only prove meaningful when sufficient in vitro studies have been completed along with the necessary clinical outcome evaluations.

In efforts to integrate clinical microbiologic data, that is, antimicrobial susceptibility results and pharmacologic data, programs have been developed under the banner of "antimicrobial stewardship" that are designed to "monitor and direct antimicrobial use at a health care institution, thus providing a standard evidence-based approach to judicial antimicrobial use" (20,21). Outcome measures that are desired include improved patient outcome, improved safety, reduced resistance, and reduced cost (22). These measures may be difficult to assess as well as difficult to achieve. Within the frame of "antimicrobial stewardship program" (ASP), the use of clinical pharmacists and the electronic health record offer a streamlined method for implementation (23,24). Moreover, new approaches in rapid pathogen detection and identification can prove highly effective when integrated into an ASP (25).

THE HUMAN AND ANIMAL MICROBIOME

The microbiome, whether animal or human, can be defined as the aggregate genomes of their respective microbiota and the varied metabolic activities which they encode. The accumulated information has the potential to revolutionize the way we view contemporary therapeutics. During the past century, the information that has been gathered in the area of pharmacology, describing rates of absorption, distribution, metabolism, and excretion for hundreds of compounds—in our case, antibiotics—referred to today as xenobiotics (compound foreign to a living organism, which include antimicrobial agents as well as other therapeutic drugs). As yet a poorly understood component of xenobiotic metabolism is the effect-impact of the vast number (trillions, 10^7) of microorganisms that reside in the gastrointestinal tract. Although the discovery of antibiotics is decades old, spanning the past century, it appears we are at the beginning of determining the "intended" collateral damages that antibiotics can impart on the symbiotic microorganisms living in our gastrointestinal tract (26). The gathering of the varied and numerous microbial members within our person play vital roles in the maintenance of human health by freeing nutrients and/or energy from otherwise inaccessible dietary substrates, promoting the differentiation of host cells and tissues, stimulating the immune system, and protecting the host from invasion by pathogens. The assemblage of

human-associated microbial communities does not generally proceed smoothly. There are several examples where some fraction of the community is removed or killed (e.g., oral hygiene). The effect of antimicrobial agents on the gut microbiota serves as a model for disturbance in human-associated communities. It is estimated, that on any given day, 1% to 3% of people in the developed world are exposed to pharmacologic doses of antibiotics (27).

Antimicrobial therapy is intended to achieve sufficient drug concentration for a sufficient duration in a particular body compartment so that the targeted pathogen is eliminated. Even if this aim were always attained, the antibiotic will also be found at varied concentrations of several locations within the body depending on the mode of administration and PK properties. When members of the microbiota are exposed to antibiotics that affect their growth rate without killing them, there is selection for resistance. The horizontal transfer of antibiotic-resistant determinants takes place in the human gut and oral communities and their reservoir serves as the starting point/place for transfer to pathogens as well as the resident microbiota. The collateral damage to the human microbiome exerted by contemporary antimicrobials through overuse and extended spectrum has likely been the driving force behind the proliferation of MDROs and members of the ESKAPE group. Understanding the balance and fragility of the human microbiome so as to use "microbiome-sparing antimicrobial therapy," develop techniques to restore and maintain the indigenous microbiota, as well as use protective mechanisms encoded by an intact microbiome will limit the expanding scope of resistance (28).

As we decipher the heterogeneous environment of the human body/microbiome, we recognize that microorganisms encounter these environments replaced with transient chemical and nutrient gradients. Clinically, antibiotic gradients develop when a patient begins, ends, or neglects a prescribed regimen. To simulate in vivo conditions, Zhang et al. (29) constructed a microfluidic device consisting of a tiny chamber device. The investigators then determined the effect of the microenvironment generated within the chambers on bacterial populations grown in them. They found that when the test organism *Escherichia coli* is grown in a heterogeneous environment that contains a steep antibiotic concentration, ciprofloxacin in their simulated experiments, they demonstrated rapid and repeatable acquisition and fixation of ciprofloxacin mutations compared with bacteria grown in homogeneous environments. If we suppose that some parts of the human body resemble heterogeneous environments rather than the in vitro containment of a Petri dish and a flask, then the environment proposed by Zhang et al. (29) would provide a more relevant model for the development of antimicrobial resistance.

IMPACT OF NEW TECHNOLOGIES

More than 10 years have elapsed since the first polymerase chain reaction (PCR) assays for antimicrobial resistance was evaluated. For this event, the assay was directed to MRSA. Few targeted assays have followed but have included vancomycin resistance in *Enterococcus* spp and rifampin resistance in *Mycobacterium tuberculosis*. To date, there has not been a developed panel/array of molecular susceptibility testing for several common drug resistance mechanisms.

A barrier for molecular susceptibility testing has been the characterization of mutation(s) associated with the resistance phenotype and the subsequent development of tests specific for these markers. For example, mutants generated during in vitro selection of antimicrobial-resistant strains can differ from those that develop naturally in human populations and cause clinical disease (30).

By far, in the second decade of the 21st century, the greatest need and challenge in molecular assay development is the capability to detect resistance determinants among the gram-negative bacilli. In the family Enterobacteriaceae, several hundred mechanisms have been reported causing resistance to β-lactams, cephalosporins, monobactams, and/or carbapenems. Among Enterobacteriaceae, β-lactam resistance has been attributed to several mechanisms, which include ESBLs, AmpCs, metallo-β-lactamases (MBLs), and KPCs. The number of genotypically unique ESBLs total more than 200 (31).

Directed detection of the several and as yet uncovered resistance mechanism requires technology with efficient and specific methodology capable to be multiplexed beyond that of PCR platforms extant and will probably be based on microarrays, metabolite detection assays, or direct sequencing. Key to the widespread acceptance of these newer technologies will be the evidence to demonstrate the high negative predictive values and the associated sensitivity to detect low levels of gene expression.

Which technologies of those that are currently in use will prove meaningful for future development is unknown. Although real-time PCR has revolutionized clinical molecular diagnostics, permitting detection of targets in a closed system within a 45-minute to 2-hour time frame, an associated limitation is the number of fluorophores that can be used for simultaneous detection of multiple targets—usually six. For detection of a multiplicity of targets (more than six), liquid- and solid-phase microarrays may best suit this requirement. Examples of this technology would be XTAG (Luminex, Austin, TX) or BeadExpress (Illumina, San Diego, CA). Solid arrays which have been in use for several years in research laboratories as represented by Nanosphere, Inc. (Northbrook, IL) are an alternative. Other solid array systems such as GeneChip (Affymetrix, Santa Clara, CA) and BioFilmChip (Autogenomics, Vista, CA) are other alternatives.

Recent advances in nucleic acid sequencing technology have made sequencing the entire human genome—or for this discussion, the microbial code—both technically and economically feasible. In clinical medicine, WGS has been heralded to clarify molecular diagnosis and guide therapy—giving rise to the concept of personalized medicine. Several benchtop, high-throughput sequencing platforms no larger than our all-in-one printer are available for this function. Among them, the 454 GS Junior (Roche, Branford, CT), MiSeq (Illumina, San Diego, CA), and the Ion Torrent PGM (Life Technologies, Carlsbad, CA) offer modest setup and operating costs. Each instrument can generate sufficient data for a draft bacterial genome (32). It is not unlikely that given the multiple bioinformatic methods available, the capability to analyze the information encoded within the complete genome sequence for determining antimicrobial resistance would be available. In the context of this discussion, reference is made to using the decoded microbial genome to identify resistance markers that would redirect health care providers from treating patients with those drugs that would predictably become ineffective when the microbial target would produce the inactivating enzymes or processes that would render the antiinfective useless. Although the time required to accomplish this is rapid (days) compared to earlier sequencing iterations, it does not meet the clinical needs for "rapid" diagnosis. Each instrument can generate the data required for a draft bacterial genome sequence suitable for identifying and characterizing pathogens.

Deciphering DNA sequences is essential for virtually all areas of biologic investigation. The classical capillary electrophoresis (CE)–based sequencing has enabled the elucidation of genetic information in almost any organism or biologic system. In order to overcome inherent barriers in

this experimental system, a fundamentally different technology was developed—next-generation sequencing (NGS). NGS is especially suited for microbial systems as it has the capability to evaluate alterations throughout the genome without prior knowledge and is therefore adaptable for unculturable microorganisms. NGS has increased the rate of data output each year since its inception in 2007 so that in 2012, 1 terabase (Tb) of data is available in a single sequencing run compared to 1 gigabase (Gb) in 2007. Associated with this exponential increase in output is a 10^5-fold decrease in the cost of determining the genome of a microorganism. In 1995, sequencing the 1.8-megabase (Mb) genome of *Haemophilus influenzae* with CE technology costed approximately $1 million and took about 1 year. Sequencing the 5-Mb genome of *E. coli* in 2012 with NGS technology can be accomplished in 1 day at a cost of about $100.

The molecular detection of resistance is an attractive concept; however, it fails to direct or recommend any specific treatment plan, antiinfective, or course of action. Genotypic resistance approaches have been used throughout the development of antiretroviral agents to monitor the treatment of HIV. But this was necessitated as the routine phenotype testing for susceptibility (or resistance) was not readily accessible to clinical laboratories because of biosafety precautions necessary when dealing with the human immunoproliferative agent—HIV. As is the case in WGS, the issue will become for microbial targets, the capability of dedicated software to interrogate the decoded microbial sequence to identify resistant markers for extant antimicrobial agents.

Recent reports have demonstrated how high-throughput genome sequencing of bacterial genomes were used to monitor disease spread and control infections in hospital settings. These investigations were associated with an outbreak of carbapenem-resistant *K. pneumonia*, which occurred at the U.S. National Institute of Health Clinical Center (33); cases of group A streptococcus (GAS; *Streptococcus pyogenes*) isolates associated with outbreaks of puerperal sepsis in Australian hospitals (32); and an outbreak of MRSA in a neonatal intensive care unit at a hospital in Cambridge, United Kingdom (34). These three studies show the future direction of clinical laboratory studies, which enable same-day diagnosis antibiotic resistance gene profiling and virulence gene detection.

A departure from traditional molecular diagnostics for targeting either DNA or RNA that encode resistance determinants would encompass the identification of proteins responsible for resistance—the field of proteomics. Advances in mass spectrometry describe matrix-associated laser desorption-time of flight (MALDI-TOF) identification of bacteria. This technique establishes the protein signature that can fingerprint the identification of clinically significant bacteria; however, direct detection of resistance determinants has not been established because several proteins can be involved in drug resistance (35).

As antibiotic resistance mechanisms among pathogenic microorganisms, especially the Enterobacteriaceae, are discerned, there is compelling need to rapidly and definitively identify them. Advanced diagnostics employing molecular methods is considered to be a key driver to improve therapeutic outcome—molecular arrays and NGS are key to providing the most promising opportunities.

REFERENCES

1. Boucher HW, Talbot GH, Bradley JS, et al. Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. *Clin Infect Dis* 2009;48:1–12.
2. European Centre for Disease Prevention and Control/European Medicines Agency Joint Technical Report. The bacterial challenge: time to react. http://www.ema.europa.eu/docs/en_GB/document_library/Report/2009/11/WC500008770.pdf. Updated September 2009. Accessed May 17, 2014.
3. Rice LB. Federal funding for the study of antimicrobial resistance in nosocomial pathogens: no ESKAPE. *J Infect Dis* 2008;197:1079–1081.
4. Tillotson GS. Stimulating antibiotic development. *Lancet Infect Dis* 2010;10:2–3.
5. Infectious Diseases Society of America. The 10 × '20 initiative pursuing a global commitment to develop 10 new antibacterial drugs by 2020. *Clin Infect Dis* 2010;50: 1081–1083.
6. Wright GD. The antibiotic resistome: the nexus of chemical and genetic diversity. *Nature Rev Microbiol* 2007;5: 175–186.
7. D'Costa VM, King CE, Kalan L, et al. Antibiotic resistance is ancient. *Nature* 2011;477:457–461.
8. Sommer MOA, Dantas G, Church GM. Functional characterization of the antibiotic resistance reservoir in the human microflora. *Science* 2009;325:1128–1131.
9. Dantas G, Sommer MOA, Oluwasegun RD, et al. Bacteria subsisting on antibiotics. *Science* 2008;3201:100–103.
10. D'Costa VM, McGrann KM, Hughes DW, et al. Sampling the antibiotic resistome. *Science* 2006;311:374–377.
11. Garnacho-Montero J, Garcia-Garmendia JL, Barrero-Almodora A, et al. Impact of adequate empirical antibiotic therapy on the outcome of patients admitted to the intensive care unit with sepsis. *Crit Care Med* 2003;31: 2742–2751.
12. Vallees J, Rello J, Ochagavia A, et al. Community-acquired bloodstream infection in critically ill adult patients: impact of shock and inappropriate antibiotic therapy on survival. *Chest* 2003;123:1615–1624.
13. Ibrahim EH, Sherman G, Ward S, et al. The influence of inadequate antimicrobial treatment of bloodstream infections on patient outcomes in the ICU. *Chest* 2000;118: 145–155.
14. Rello J, Gallego M, Mariscal D, et al. The value of routine microbial investigation in ventilator-associated pneumonia. *Am J Respir Crit Care Med* 1997;156:196–200.
15. Alvarez-Lerma F. Modification of empiric antibiotic treatment in patients with pneumonia acquired in the intensive care unit. ICU-Acquired Pneumonia Study Group. *Intensive Care Med* 1996;22:387–394.
16. Rex JH, Pfaller MA. Has antifungal susceptibility testing come of age? *Clin Infect Dis* 2002;35:982–989.
17. Sakoulas G, Moise-Broder PA, Schentag J, et al. Relationship of MIC and bactericidal activity to efficacy of vancomycin for treatment of methicillin-resistant *Staphylococcus aureus* bacteremia. *J Clin Micro* 2004;42: 2398–2402.
18. Centers for Disease Control and Prevention. Effects of penicillin susceptibility breakpoints for *Streptococcus pneumoniae*. United States, 2006-2007. *MMWR Morb Mortal Wkly Rep* 2008;50:1353–1355.
19. Tam VH, Gamez EA, Westan JS, et al. Outcomes of bacteremia due to *Pseudomonas aeruginosa* with reduced susceptibility to piperacillin-tazobactam: implications on the appropriateness of the resistance breakpoint. *Clin Infect Dis* 2008;46:862–867.
20. Delit TH, Owens RC, McGowan JE Jr, et al. Infectious Diseases Society of America and the Society for Healthcare Epidemiology of America guidelines for developing an institutional program to enhance antimicrobial stewardship. *Clin Infect Dis* 2007;44:159–177.
21. Tamma PD, Cosgrove SE. Antimicrobial stewardship. *Infect Dis Clin North Am* 2011;25:245–260.
22. McGowan JE. Antimicrobial stewardship—the state of the art in 2011: focus on outcome and methods. *Infect Control Hosp Epidemiol* 2012;33:331–337.
23. Salmasian H, Freedberg DE, Abrams JA, et al. An automated tool for detecting medication overuses based on the electronic health records. *Pharmacoepidemiol Drug Saf* 2013;22:183–189.
24. Linsky A, Simon SR. Medication discrepancies in integrated electronic health records. *BMJ Qual Saf* 2013;22: 103–109.
25. Perez KK, Olsen RJ, Musick WL, et al. Integrating rapid pathogen identification and antimicrobial stewardship significantly decreases hospital costs. *Arch Pathol Lab Med* 2013;137:1247–1254.

26. Blaser M. Antibiotic overuse: stop the killing of beneficial bacteria. *Nature* 2011;476:393–394.
27. Goossens H, Ferech M, Vander Stichele R, et al. Outpatient antibiotic use in Europe and association with resistance: a cross-national database study. *Lancet* 2005;365:579–587.
28. Tosh PK, McDonald LC. Infection control in the multidrug-resistant era: tending the human microbiome. *Clin Infect Dis* 2012;54:707–713.
29. Zhang Q, Lambert G, Liao D, et al. Acceleration of emergence of bacterial antibiotic resistance in connected microenvironments. *Science* 2011;333(6050):1764–1767.
30. Piatek AS, Telenti A, Murray MR, et al. Genotypic analysis of *Mycobacterium tuberculosis* in two distinct populations using molecular beacons: implications for rapid susceptibility testing. *Antimicrob Agents Chemother* 2000;44:103–110.
31. Leinberger DM, Grimm V, Rubtsova M, et al. Integrated detection of extended-spectrum-beta-lactam resistance by DNA microarray-base genotyping of TEM, SHV, and CTX-M genes. *J Clin Microbiol* 2010;48:460–471.
32. Loman NJ, Misra RN, Dallman TJ, et al. Performance comparison of benchtop high-throughput sequencing platforms. *Nature Biotechnol* 2012;30(5):434–439.
33. Ben Zakour NL, Venturini C, Beatson SA, et al. Analysis of *Streptococcus pyogenes* peripheral sepsis cluster by use of whole-genome sequencing. *J Clin Microbiol* 2012;50: 2224–2228.
34. Köser CU, Holden MT, Ellington MJ, et al. Rapid whole-genome sequencing for investigation of a neonatal MRSA outbreak. *N Engl J Med* 2012;366:2267–2275.
35. Carapetis JR, Steer AC, Mulholland EK, et al. The global burden of group A streptococcal disease. *Lancet Infect Dis* 2005;5:685–694.
36. Shah NH, Gharbia SE, eds. *Mass spectrometry for microbial proteomics*. New York: John Wiley and Sons, 2010.

Chapter 1

The Breakpoint

Matthew A. Wikler, Franklin R. Cockerill III, and Paul G. Ambrose

DEFINITION AND CLINICAL UTILITY OF ANTIMICROBIAL BREAKPOINTS

A breakpoint, in its simplest terms, represents the concentration of an antimicrobial agent that separates populations of microorganisms. Breakpoints are used in many ways, and so there may be more than one breakpoint for a specific antimicrobial agent–microorganism combination. It is also of interest that a breakpoint may change from time to time for a variety of reasons, as discussed later. In addition, breakpoints can vary from one country to another and from one official body to another in the same country. For example, in the United States, both the Clinical and Laboratory Standards Institute (CLSI), formerly the National Committee for Clinical Laboratory Standards (NCCLS), and the U.S. Food and Drug Administration (FDA) may provide breakpoints for the same antimicrobial agents. Additionally, breakpoints in the European Union (EU) are set by the European Committee on Antimicrobial Susceptibility Testing (EUCAST). The determination of a specific breakpoint is not a black-and-white decision, as many factors must be considered when selecting breakpoints.

To assist physicians in selecting antimicrobial agents to treat patients, clinical microbiologists categorize clinical isolates as drug susceptible, drug intermediate, or drug resistant. A result of "susceptible" assumes that an infection due to the isolate may be appropriately treated with the dosage of an antimicrobial agent recommended for that type of infection and infecting species. A result of "resistant" assumes that the isolate is not inhibited by the usually achievable concentrations of the agent with normal dosage schedules and/or falls in the range where specific microbial resistance mechanisms are likely and clinical efficacy has not been reliably attained in treatment studies. A result of "intermediate" assumes that an infection due to the isolate may be appropriately treated in body sites where the drugs are physiologically concentrated or when a high dosage of the drug can be used. The category of intermediate is also used as a "buffer zone" to prevent small, uncontrolled technical factors from causing major discrepancies in interpretations (1).

Ultimately, the purpose of breakpoints is to provide clinicians with information to assist in making decisions about antimicrobial treatments for patients with infections. Breakpoints serve many purposes, some of which are important for an individual patient, others for epidemiologic reasons. If breakpoints did not result in better patient care, then there would be little need to determine them other than as an academic exercise.

The remainder of this chapter focuses on breakpoints for bacteria, as these are currently the most advanced; however, many of the principles apply to the setting of breakpoints for other types of microorganisms (e.g., fungi).

HISTORICAL EVOLUTION AND CURRENT CRITERIA FOR ESTABLISHING BREAKPOINTS

CLSI has been providing standards for the testing of bacteria and breakpoints since 1975. Initially, breakpoints were established by examining scatterplots of the distributions of bacterial isolates versus the results of susceptibility testing conducted with antibacterial agents. Such scatterplots would frequently divide the bacterial isolates into two populations, one of which would appear to be more susceptible to the antibacterial agent being tested, the other less susceptible. The breakpoint would be the drug concentration separating the two populations. Establishing breakpoints in such a manner is probably suitable for epidemiologic purposes, as it allows one to easily determine shifts in the populations of organisms and to identify the emergence of resistant populations. From a clinical perspective, however, such an approach does not take into account the clinical implications.

In an attempt to improve the process for establishing breakpoints, CLSI has provided specific guidelines contained in a special document. The first version of this document was published in 1994 (2) and introduced the concept of looking at other types of data, including clinical data, in an attempt to correlate proposed breakpoints with what is likely to occur in the clinical setting. A revision of this document was published in 2001 (3). At the current time, so-called clinical breakpoints (antimicrobial susceptibility test interpretive categories) are determined by CLSI utilizing the following types of information: microbiologic data, animal modeling data, pharmacokinetic (PK) and pharmacodynamic (PD) modeling data, and human clinical data. These data are all considered and compared one against the other. In an ideal world, these data would all correlate with one another so that a breakpoint could be determined with certainty.

The microbiologic data considered consist of distributions of bacterial isolates and their minimum inhibitory concentrations (MICs). Numerous distributions are evaluated including those for a broad spectrum of organisms against which the antimicrobial agent is likely to be utilized and those for select populations of organisms that have specific types of resistance mechanisms. As clinical studies are conducted with a new antimicrobial agent, the susceptibility patterns observed in actual patients enrolled in studies are also reviewed. By utilizing data of this type, one can gain a sense of the various populations of organisms that exist and their relative susceptibility to the antimicrobial agent. Animal studies are quite useful in determining which pharmacokinetic-pharmacodynamic (PK-PD) measure one should be evaluating when trying to predict clinical efficacy. The three most common PK-PD measures are the duration of time the drug concentrations remain above the MIC (T>MIC), the ratio of the maximal drug concentration to the MIC (C_{max}:MIC ratio), and the ratio of the area under the concentration time curve at 24 hours to the MIC (AUC_{0-24}:MIC ratio). For most classes of antimicrobial agents, animal studies have demonstrated the ability to predict clinical efficacy by examining specific parameters (4). For example, it has been clearly demonstrated for β-lactam antibiotics that the most critical parameter predictive of clinical

outcomes is the time that free drug plasma concentrations remain above the MIC of the causative organism (5–7). This is an example of a class of antibiotics where the predictive parameter is "time dependent." For cephalosporin antibiotics and *Streptococcus pneumoniae*, it appears that clinical success is likely if the free drug concentration above the MIC of the causative organism is maintained for 40% to 50% of the dosing interval, while for penicillins, the target appears to be 30% to 40% of the dosing interval (4).

The area under the drug concentration time curve (AUC) is a measure of drug exposure. Mathematically, the AUC is calculated as the integral of the drug concentration time curve. Response to drugs in vivo can usually be linked to the AUC. In some instances, the shape of the concentration time curve can affect in vivo response to a drug, and thus other measures of exposure (e.g., C_{max}, C_{min}) can also be important. Fluoroquinolone antibiotics have been demonstrated in animal models to be "concentration dependent"; that is, clinical outcomes can be predicted based on the AUC:MIC ratio and/or by the C_{max}:MIC. For the fluoroquinolones, it appears that clinical success (8,9) depends on attaining a free drug AUC:MIC ratio of around 30 for gram-positive organisms and around 100 for gram-negative organisms.

Although these general PK-PD targets tend to apply to many types of infections, one must keep in mind that levels obtained in certain tissues and body fluids may result in these targets not being predictive. For example, for drugs that are excreted by the kidneys and where active drug is concentrated in the urine, one would anticipate the ability to successfully treat organisms in the urinary tract with higher MICs. On the other hand, most drugs do not achieve high levels in the cerebrospinal fluid (CSF), and so one would anticipate that higher doses of an antimicrobial agent may be required to adequately treat an infection in that site. It would not be sufficient only to determine PK-PD targets based on responses in animal models, and so it is important that the results of clinical studies be correlated with these targets. Such work has been done for many classes of antimicrobial agents (4,8,10–14). Unfortunately, for new classes of antimicrobial agents, including LpxC inhibitors (deacetylase inhibitors of endotoxin biosynthesis), topoisomerase type-B subunit inhibitors, β-lactam–β-lactamase inhibitors, the correlations between the targets and the clinical outcomes in humans have yet to be well studied.

Once the PK-PD measure and target predicting clinical success has been identified, one can integrate this information with human PK data to estimate the probability of attaining drug exposures sufficient across a range of MIC values. One of the best ways to conduct such an analysis is by using Monte Carlo techniques (15,16). Basically, the strategy is to take (a) the PK parameters along with anticipated variability and (b) the MIC values of organisms likely to cause an infection along with the proportion of time; a specific MIC value is achieved or exceeded by in vivo concentrations of antimicrobial agent and then model patients by randomly matching up PK profiles and MICs. By using such a technique, one can easily simulate 5,000 or 10,000 patients and make predictions as to what MICs one is likely to be able to treat successfully with various dosing regimens (17,18). Ideally, this process should be accomplished in the earliest stages of drug development, as this allows one to determine the optimal dosing regimen likely to result in a successful clinical outcome while minimizing the potential for toxicity. Such PK-PD modeling can also be utilized to justify the initial breakpoint for a new antimicrobial agent prior to the availability of a large amount of clinical data (19,20).

Ultimately, the purpose of breakpoints is to provide information to the clinician for the selection of optimal antimicrobial therapy. Because of this, it is critical to evaluate clinical data from well-designed clinical studies which correlate breakpoints with clinical outcomes. Unfortunately, this is far from an exact science, as there are many factors that determine clinical outcomes other than the antimicrobial agent used. Consequently, clinical correlations are generally used to confirm susceptibility breakpoints predicted by the previously mentioned techniques and data.

For many reasons, the true limits of an antimicrobial agent are rarely tested in clinical studies conducted for the purpose of gaining regulatory approval. First, most of these studies exclude or discontinue patients whose infections are caused by organisms with an MIC above a tentative breakpoint. As a result, even if clinical studies could demonstrate a breakpoint, the probability of this happening is greatly reduced. In most cases, clinical studies can be ethically designed in a manner that would blind the investigator to susceptibility test results, allowing the decision to continue or discontinue therapy with the study drug to be determined by clinical and microbiologic responses. Clinical studies so designed are more likely to aid in determining a clinical breakpoint. Another reason that breakpoints often fail to be determined by clinical data is that many of the newer antibiotics being developed are quite potent and only a small percentage of organisms will have MICs high enough to truly test their limits. In fact, few or no patients may be enrolled who have infections due to organisms with MICs at a sufficient level to uncover the limits of the antibiotic being evaluated.

ESTABLISHING BREAKPOINTS BY SITE OF INFECTION

It is sometimes necessary to consider the need for different breakpoints based on the site of the infection for which the antimicrobial agent is intended. For example, the ability of a drug to concentrate within the CSF is frequently quite different from the ability of the same drug to be concentrated in the urine or into tissues such as lung. As a result, it is reasonable to anticipate the requirement for a different breakpoint for the treatment of meningitis, urinary tract infections, or pneumonia.

As an example, CLSI recommends different penicillin breakpoints for *S. pneumoniae* isolated from spinal fluid as compared with respiratory secretions. Due to PK/PD and outcome data, lower (more stringent) breakpoints are provided for CSF isolates versus higher (more lenient) breakpoints for respiratory isolates (13).

ESTABLISHING BREAKPOINTS WHEN THERE ARE NO RESISTANT BACTERIAL STRAINS

As previously noted, many of the newer antimicrobial agents being developed are relatively potent and few resistant bacterial strains exist. When there is a dearth of resistant strains, it is difficult to obtain animal or clinical data to determine the true breakpoint. In these circumstances, the breakpoint is generally set at one or two 2-tube dilutions above the known susceptible population of strains for that organism. In such a situation, only a susceptible and not a resistant breakpoint is usually published, along with a notation that any strains isolated with a higher MIC should be sent to a reference laboratory to confirm the results. With time, a population of less susceptible strains will frequently emerge, and additional animal and clinical data may become available. At that point, it may become possible to establish a breakpoint reflective of the new situation.

CORRELATION BETWEEN MINIMAL INHIBITORY CONCENTRATION AND DISK ZONES

Many laboratories do not do MIC testing but rather depend on disk diffusion susceptibility testing methods. In order to meet the needs of these institutions, breakpoints are set for disk diffusion methods by correlating MIC results and disk diffusion results. Once again, various types of MIC distributions of clinical isolates of bacteria are reviewed, including distributions for a broad spectrum of organisms against which the antimicrobial agent is likely to be used and for select populations of organisms that have specific types of resistance mechanisms. Statistical methods are generally utilized to determine the best correlation between disk zones and MICs. Once the disk zone breakpoint is statistically determined, the rates of discrepant results are evaluated (i.e., where one method predicts susceptible and the other predicts resistant or intermediate). The number of discrepancies that occur within one twofold dilution of the intermediate MIC is less important than the number of discrepancies that occur at other MICs. After analysis of such discrepancies, the disk test may be adjusted to make it more predictive of the MIC test (i.e., by reducing the number of discrepancies). In some cases, it is impossible to develop a disk test that correlates with the MIC test. A recommendation is then made to not perform disk testing.

OVERRIDING THE BREAKPOINT

There are circumstances in which the breakpoint determined utilizing the standard methods is known to be inaccurate. When this occurs, the laboratory is instructed to override the results of the test and to adjust the report to the physician. For example, in recent years, gram-negative organisms that produce extended-spectrum β-lactamases (ESBLs) emerged. The standard testing methods that were used produced results indicating that the organism was susceptible to cephalosporins; however, because of the presence of an ESBL, these drugs were ineffective against such strains. As a result, CLSI developed specific testing methods to detect ESBL-producing strains. When such strains were detected using these specific methods, laboratories were instructed to override all MIC results previously interpreted as susceptible with the interpretation of resistant (21). Recently, CLSI has modified the MIC breakpoints for these drugs so that ESBL-producing strains are essentially captured. However, until these new breakpoints are adopted by laboratories, the specific ESBL tests must still be used with "overriding of results" as directed. As new mechanisms of resistance develop, it is critical that organizations and agencies that produce standardized antimicrobial susceptibility testing methods and interpretive criteria be diligent in looking for circumstances where the results of such tests are not accurate.

THE USE OF SUSCEPTIBILITY TESTS OF ONE ANTIBIOTIC TO PREDICT THE SUSCEPTIBILITY OF ANOTHER ANTIBIOTIC

Laboratories often use testing systems developed by antimicrobial susceptibility testing manufacturers. Such testing systems may contain a panel of antibiotics that do not replicate the available agents in a particular hospital or within the formulary of a particular health care system. In such a case, a laboratory may wish to use the results for one antibiotic to predict the susceptibility of an organism to another similar antibiotic. This has been a common practice, for example, with various cephalosporin antibiotics. In Table 1 of CLSI document M100 (21), there are suggestions as to when this may be possible. One must be aware that the correlations for certain types of organisms may be much better than for others within the same antibiotic class and that depending on one antimicrobial agent to predict another will invariably lead to some reporting errors.

WHY ARE THERE DIFFERENT BREAKPOINTS IN VARIOUS PARTS OF THE WORLD?

It is not uncommon to find that the clinical breakpoints for an antimicrobial agent–microorganism combination are different in different parts of the world. The reasons for this should become clear when one examines the variables involved in determining breakpoints. Breakpoints are set based on results achieved using a standardized testing method. If everyone used exactly the same testing method with adequate controls, the results would be expected to be similar. Unfortunately, testing methods are not currently standardized around the world, and thus there is the potential for different breakpoints to be established using different methods. Second, an antimicrobial agent might be used differently in different geographic areas. If it is customary to use an antimicrobial agent at a higher dose or to dose more frequently (including constant infusion) in one geographic area, the breakpoint will likely be higher in that area. Third, different microorganisms are encountered in different parts of the world. If resistant strains of bacteria are present in one geographic area but not other areas, it may be necessary to regionally adjust the breakpoints to ensure that the new resistant strains are being properly reported. There are also public health reasons why a breakpoint may vary. Public health authorities in one geographic area may want to avoid a resistance problem, deal with a resistance problem, or promote the use of certain antimicrobial agents over others. One way to impact antimicrobial use is to adjust breakpoints.

CURRENT EFFORTS TO DEVELOP STANDARDIZED METHODS AND BREAKPOINTS IN OTHER PARTS OF THE WORLD

Currently, efforts are being undertaken in various parts of the world to develop standardized methods for susceptibility testing. Ultimately, it would be ideal if one standardized testing method was accepted worldwide, as this would allow the direct comparison of results from one part of the world to another. The lack of harmonization of methods can create significant problems when evaluating epidemiologic trends. For example, suppose the goal is to examine the development of resistance for a particular organism and its spread in various parts of the world. The use of different methods makes it impossible to ascertain the true level of resistance. Even if one standardized testing method was accepted and utilized throughout the world, it is likely that there would still be different clinical breakpoints, for the reasons noted previously.

Another problem resulting from the lack of standardized methods for susceptibility testing concerns the development of new antibiotics. When conducting clinical trials and looking for correlations between outcomes and MICs or disk zones, it is necessary to use the same methods wherever the drug is tested.

The EUCAST is a standing committee of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID). EUCAST was set up to standardize susceptibility testing in Europe so that comparable results and interpretations would be produced. It has both a general committee, whose membership includes representatives from all European countries, from the pharmaceutical industry, and from the in vitro media

and device industries, and an ESCMID-appointed steering committee, which consists of a chair, a scientific secretary, six National Breakpoint Committee representatives, and two representatives from the general committee. Decisions are made by the steering committee after consultation with the general committee (ESCMID Web site, https://www.escmid.org). Unlike the CLSI, which develops only clinical breakpoints, EUCAST is in the process of developing both epidemiologic and clinical breakpoints. Epidemiologic breakpoints are breakpoints that differentiate the wild-type strains from strains that have developed resistance. Epidemiologic breakpoints can be extremely valuable when one wants to evaluate the emergence of resistant populations of organisms. The methods used by EUCAST (22,23) are in general agreement with those of the CLSI. EUCAST has been collecting MIC distribution data from worldwide sources for the purposes of establishing epidemiologic breakpoints. This extensive database is available on the EUCAST Web site (www.eucast.org).

WHY THE BREAKPOINTS OF CLSI AND THE FDA MAY DIFFER

At times, breakpoints contained in the FDA-approved package insert for an antimicrobial agent do not agree with the breakpoints published in CLSI documents. There are many reasons why this may occur, including differences in the interpretation of data and differences in how the two organizations function. When a new antimicrobial agent is approved by the FDA, interpretive criteria utilizing the CLSI standardized methods are approved and included in the product label. Most pharmaceutical sponsors will also submit a package of data to CLSI within a year of a new drug approval requesting breakpoints. Although there is general agreement between the FDA and CLSI, at times there are differences based on interpretation of the data. It is common for differences to occur after an antimicrobial agent has been on the market for a few years, as new resistance mechanisms become apparent requiring a reevaluation of the breakpoint. Unlike the FDA, CLSI has the ability to review breakpoints for any antimicrobial agent when there appears to be a need to do so. As a result, CLSI will make changes in single drugs or will frequently evaluate a class of drugs at the same time and make whatever adjustments seem necessary. Currently, the FDA generally considers a change in a breakpoint only when the sponsor submits a package to the FDA requesting a change. FDA staff participate in CLSI meetings as advisors and reviewers. Sponsors are encouraged to submit new data to the FDA to allow for updating the product label.

The obvious question arising from this is which breakpoints will clinical laboratories in the United States use? When there are published CLSI breakpoints, US laboratories use these breakpoints when testing organisms and providing reports to physicians. In fact, US laboratories are tested and accredited based on their compliance with CLSI methods and interpretive standards. If US laboratories use devices provided by in vitro diagnostic (IVD) manufacturers, those manufacturers must receive FDA approval at the breakpoints the FDA specifies before marketing these devices.

THE IMPORTANCE OF QUALITY CONTROL

The ability of the laboratory to follow the standardized testing methods utilized for setting breakpoints and the ability of the automated testing system to replicate the results that would be obtained utilizing the standard methods are critically important. Clearly, if such methods are not followed and well controlled, then the MIC or disk zone reported may be inaccurate, potentially resulting in a misinterpretation and inaccurate reporting to the health care provider. For this reason, care must be taken in performing these tests, and proper quality control must be implemented. In order to help the laboratory, CLSI and other organizations that produce documents outlining standardized methods provide quality control ranges for various standard bacterial strains tested against specific antibiotics. It is critical to ensure that the test performed on the quality control strains produces results that are within the accepted ranges. In addition, the laboratory must make certain that the breakpoints applied are those based on the methods that are being utilized. It is also critical that growth of the bacterial strain is sufficient for an accurate MIC or disk zone to be obtained. That is why it is necessary to have a control well or area on the test plate where the bacterial strain can grow uninfluenced by the antimicrobial agent.

HOW TO REPORT AND USE BREAKPOINTS

Although breakpoint information is valuable when used for a specific patient, it can also have an impact on antimicrobial agent selection for a much larger group of patients. Most antimicrobial agent use is empiric; that is, a patient appears with what seems to be a bacterial infection, and the physician prescribes an antimicrobial agent without knowledge of the causative organism or its susceptibility. If the laboratory periodically collects its susceptibility testing data, summarizes such data, and distributes them to its physicians, then physicians are in a better position to prescribe antimicrobial agents likely to be successful. Most hospitals publish an antibiogram once or twice a year just for this purpose. There are numerous things one must consider when constructing antibiograms, and CLSI document M39-A3 (24) provides a guideline to help laboratories in developing them.

RESETTING BREAKPOINTS

Although epidemiologic breakpoints tend to be static, clinical breakpoints are not. There are numerous reasons why a breakpoint may need to be changed, and many of them are outlined in CLSI document M23 (3):

1. Strains less susceptible and/or more resistant to an antimicrobial agent may evolve.
2. Organisms with new mechanisms of resistance may develop.
3. New dosages or formulations of an antimicrobial agent and/or new clinical uses may require a change.
4. New clinical and/or pharmacologic data may suggest the need for reassessment.
5. Actions by and/or data from the FDA or other regulatory authorities, the Centers for Disease Control and Prevention (CDC), the College of American Pathologists, or other sources may suggest the need for reassessment.
6. Changes in CLSI-approved reference methods may have an impact on interpretive criteria and/or quality control parameters.
7. Other in vitro testing may suggest the need for reassessment.

8. Changes may also be made when public health concerns require action in situations where clinical information is limited.

As a recent example, in January 2010, CLSI changed the breakpoints for cefazolin against Enterobacteriaceae to reflect the emergence of resistance caused by ESBLs. However, after further review of common dosing regimens, MIC distributions and PK-PD data, this correction was determined to be too severe. Therefore, in January 2011, CLSI increased the MIC concentration interpretation for resistance by one twofold dilution (25).

PUBLIC HEALTH CONSIDERATIONS

The setting of breakpoints has an impact not only on individual patients but also on public health. The breakpoints will determine how antimicrobial agents will be perceived to work against specific organisms. As a result, when a physician receives an antibiogram of the susceptibility patterns of the organisms in his or her hospital and/or the local community, the physician's antimicrobial agent use patterns may be affected. Because the vast majority of bacterial infections are treated empirically, physicians depend on the antibiogram to direct their selection of initial antimicrobial therapy. If a breakpoint is changed and the change results in a commonly used antimicrobial agent no longer appearing to be efficacious, this may lead physicians to alter their prescribing habits. As a result, one class of drugs may end up being substituted for another. This shift in antimicrobial use can have an impact on future resistance patterns in the hospital and the community. For example, a previously low MIC that defined resistance for penicillin against *S. pneumoniae* for nonmeningitis infection resulted in a greater use of vancomycin. The increased use of vancomycin in hospitals appears to have led to an increase in the incidence of vancomycin-resistant enterococci. In addition, if a change in a breakpoint leads to the use of more expensive antimicrobial agents, there is an economic impact on the health care system. Clearly, there are important economic and health consequences when a breakpoint change results in the development of more problematic and difficult-to-treat organisms. These potential issues must be carefully considered when setting and/or changing breakpoints.

NEED FOR GREATER UNDERSTANDING OF BREAKPOINTS

As stated previously, the primary reason for breakpoints is to provide information to health care providers that will allow for the selection of antimicrobial agents likely to successfully treat infections. If a health care provider neither understands what the susceptibility report means nor understands the assumptions that underlie the report, then the actions taken may not be optimal for the patient. For example, if the breakpoint is set based on a specific dose of an antimicrobial agent being used, and if the physician uses a lower dose, then the actual clinical result may not be as anticipated. It is critical that efforts be made to properly communicate to health care providers what breakpoints mean and the assumptions that go into these interpretive standards. Information concerning some of the assumptions made in selecting breakpoints is contained in the documents and tables developed by CLSI and other standards-setting organizations. Unfortunately, this information rarely is communicated to physicians. If breakpoints are to be optimally utilized to maximize patient care, greater communication and education must occur. The education process could involve scientific publications that specifically discuss the decisions made by CLSI and other standards-setting organizations and the rationales for the decisions and the assumptions made. Such publications would likely be of interest primarily to microbiologists, infectious disease physicians, and hospital epidemiologists. These health care professionals should then convey the information they acquire to physicians through local educational activities.

In summary, breakpoints allow microbiology laboratories to provide valuable information to clinicians for the optimal selection of antimicrobial therapies. Epidemiologic breakpoints make it easier to detect the emergence of resistant populations of bacteria. Clinical breakpoints may vary due to differences in testing methods and in how antimicrobial agents are used in different parts of the world. Health care providers must be knowledgeable about the assumptions that go into the setting of breakpoints if they are to utilize such information to optimize patient care.

ACKNOWLEDGMENT

Ms. Tracy Dooley is thanked for her review and helpful comments for this manuscript.

REFERENCES

1. Clinical and Laboratory Standards Institute. *Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard—ninth edition*. Wayne, PA: Clinical and Laboratory Standards Institute, 2012. CLSI document M07-A9.
2. National Committee for Clinical Laboratory Standards. *Development of in vitro susceptibility testing criteria and quality control parameters*. Villanova, PA: National Committee for Clinical Laboratory Standards, 1994. NCCLS document M23-A.
3. Clinical and Laboratory Standards Institute. *Development of in vitro susceptibility testing criteria and quality control parameters; approved guideline—third edition*. Wayne, PA: Clinical and Laboratory Standards Institute, 2008. CLSI document M23-A3.
4. Craig WA. Pharmacodynamics of antimicrobials: general concept and applications. In: Nightingale CH, Murakawa T, Ambrose PG, eds. *Antimicrobial pharmacodynamics in theory and clinical practice*. New York: Marcel-Dekker, 2002:1–22.
5. Andes D, Craig WA. In vivo activities of amoxicillin and amoxicillin-clavulanate against *Streptococcus pneumoniae*: application to breakpoint determinations. *Antimicrob Agents Chemother* 1998:2375–2379.
6. Eagle H, Fleischman R, Musselman AD. Effect of schedule of administration on therapeutic efficacy of penicillin: importance of aggregate time penicillin remains at effective bactericidal levels. *Am J Med* 1950;9:280–299.
7. Eagle H, Fleischman R, Levy M. Continuous vs. discontinuous therapy with penicillin. *N Engl J Med* 1953;238:481–486.
8. Ambrose PG, Grasela DM, Grasela TH, et al. Pharmacodynamics of fluoroquinolones against *Streptococcus pneumoniae* in patients with community-acquired respiratory tract infections. *Antimicrob Agents Chemother* 2001;45:2793–2797.
9. Craig WA, Andes DR. Correlation of the magnitude of the AUC24/MIC for 6 fluoroquinolones against *Streptococcus pneumoniae* with survival and bactericidal activity in an animal model. In: Program and abstracts of the 40th Interscience Conference on Antimicrobial Agents and Chemotherapy; September 2000; Toronto, Canada.
10. Bodey GP, Ketchel SJ, Rodriguez N. A randomized study of carbenicillin plus cefamandole or tobramycin in the treatment of febrile episodes in cancer patients. *Am J Med* 1979;67:608–616.

11. Forrest A, Chodosh S, Amantea MA, et al. Pharmacokinetics and pharmacodynamics of oral grepafloxacin in patients with acute exacerbation of chronic bronchitis. *J Antimicrob Chemother* 1997;40(Suppl A):45–57.
12. Forrest A, Nix DE, Ballow CH, et al. Pharmacodynamics of ciprofloxacin in seriously ill patients. *Antimicrob Agents Chemother* 1993;37:1073–1081.
13. Preston SL, Drusano GL, Berman AL, et al. Pharmacodynamics of levofloxacin: a new paradigm for early clinical trials. *JAMA* 1997;279:125–129.
14. Ambrose PG, Bhavnani SM, Rubino CM, et al. Pharmacokinetics-pharmacodynamics of antimicrobial therapy: it's not just for mice anymore. *Clin Infect Dis* 2007;44:79–86.
15. Drusano GL. Antimicrobial pharmacodynamics: critical interactions between "drug and bug." *Nat Rev Microbiol* 2004;2:289–300.
16. Dudley MN, Ambrose PG. Pharmacodynamics in the study of resistance and establishing in vitro susceptibility breakpoints: ready for primetime. *Curr Opin Microbiol* 2000;3:515–521.
17. Ambrose PG, Craig WA, Bhavnani BM, et al. Pharmacodynamic comparisons of different dosing regimens of penicillin Gagainst penicillin-susceptible and -resistant pneumococci. In: Program and abstracts of the 42nd Interscience Conference on Antimicrobial Agents and Chemotherapy; September 27–30; San Diego, CA. Abstract A-635.
18. Dudley MN, Ambrose PG. Monte Carlo simulation and new cefotaxime, ceftriaxone, and cefepime breakpoints for *S. pneumoniae*, including strains with reduced susceptibility to penicillin. In: Program and abstracts of the 42nd Interscience Conference on Antimicrobial Agents and Chemotherapy; September 27–30; San Diego, CA. Abstract A-1263.
19. Ambrose PG, Grasela DM. The use of Monte Carlo simulation to examine the pharmacodynamic variance of drugs: fluoroquinolones against *Streptococcus pneumoniae. Diagn Microbiol Infect Dis* 2000;38:151–157.
20. Drusano GL, Preston SL, Hardalo C, et al. Use of preclinical data for selection of a phase II/III dose for evernimicin and identification of a preclinical MIC breakpoint. *Antimicrob Agents Chemother* 2001;45:13–22.
21. Clinical and Laboratory Standards Institute. *Performance standards for antimicrobial susceptibility testing; twenty-third informational supplement*. Wayne, PA: Clinical and Laboratory Standards Institute; 2013. CLSI document M100-S23.
22. EUCAST Definitive Document E. Def 2.1. Determination of antimicrobial susceptibility test breakpoints. *Clin Microbiol Infect* 2000;6:570–572.
23. EUCAST Definitive Document E. Def 3.1. Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by agar dilution. *Clin Microbiol Infect* 2000;6:509–515.
24. Clinical and Laboratory Standards Institute. *Analysis and presentation of cumulative antimicrobial susceptibility test data; approved guideline—third edition*. Wayne, PA: Clinical and Laboratory Standards Institute; 2009. CLSI document M39-A3.
25. Turnidge JD. Cefazolin and Enterobacteriaceae: rationale for revised susceptibility testing breakpoints. *Clin Infect Dis* 2011;52:917–924.

Chapter 2

Antimicrobial Susceptibility on Solid Media

John D. Turnidge and Jan M. Bell

Susceptibility testing on solid media is a widely used alternative to the traditional broth-based testing developed originally to measure minimum inhibitory concentrations. With rare exceptions, it relies on the use of agar as the solidifying agent and the nature of agar, which permits slow diffusion of chemicals through its three-dimensional matrix. There are three formats for testing using solid media: agar dilution, disk diffusion, and gradient diffusion. Of these, the one that has proven most popular and adaptable to routine laboratory testing is disk diffusion. It has been the subject of excellent chapters in previous editions of this book (1,2). The first edition of this book (3) contains an excellent chapter on some of the basic issues with testing in agar that are not fully explored here.

Of note, this chapter addresses the use of agar in susceptibility testing of conventional bacteria of human and animal origin and does not address susceptibility testing of mycobacteria, nocardiae or other aerobic actinomycetes, mycoplasmas, yeast, or molds. Methods for testing such organisms using agar have been described, and readers are referred to the standards from the Clinical and Laboratory Standards Institute (CLSI) (4–7).

FEATURES OF SOLIDIFYING AGENTS

Solidifying agents for susceptibility testing and culture media generally need the following features to be useful: (a) water solubility, (b) ability to remain solid at incubation temperatures (≤42°C), (c) ability to liquefy at higher temperatures to permit pouring and incorporation of additives, (d) chemical inertness, (e) relative transparency, and (f) resistance to bacterial degradative enzymes.

Agar

Agar is a natural product obtained from several red seaweeds from the Rhodophyceae class and related seaweeds such as *Pterocladia*, which collectively are called agarophytes. It takes its name from the Malay word *agar-agar*, which describes these seaweeds. Agar also has wide application in the food industry as a thickening and emulsifying agent. Agar liquefies when heated to boiling and does not gel until cooled to 45°C to 50°C. After gelling, it requires reheating to near boiling to liquefy again. For the preparation of bacteriologic grade agar, *Gelidium* species are almost always used because they have the preferred lower gelling temperature of 34°C to 36°C, allowing the addition of supplements (8,9).

The principal components of agar are the polysaccharides agarose and agaropectin. Purified agarose has become one of the mainstays of solid-phase electrophoretic analysis. As a natural product, agar is subject to lot-to-lot variation. Variation occurs in the presence of sulfate ions, which affect the overall negative charge of the polysaccharides, which in turn can affect diffusion of certain chemicals. Calcium is also essential in small amounts to permit gelling. Of greater importance is brand-to-brand and lot-to-lot variation in the concentrations of divalent cations, which can affect the activity of certain antimicrobials, as discussed in the following section.

Other Solidifying Agents

A range of other gelling agents have been experimented with over the years, but none has yet displaced agar for susceptibility tests. In part, this relates not only to cost but also to the daunting challenge of recalibrating end points (minimum inhibitory concentrations [MICs], zone diameters) for a very broad range of bacteria and drugs. Substances that have been examined include Separan NP10 (Dow Chemical Co, Midland, MI), a polyacrylamide that allows smaller concentrations of agar to be used; Gelrite (Merck & Co, Kelco Division, Rahway, NJ), a gellan gum formed from the fermentation products of a *Pseudomonas* species; and Neutra-Gel (Union Carbide Corp, Tuxedo, NY), a polyoxyethylene polymer that held the greatest promise when combined with a synthetic amino acid medium. Gelrite (Merck & Co, Kelco Division, Rahway, NJ) is the only one still available commercially.

FEATURES AND CHOICE OF AGAR

Since the development and promulgation of the European Committee of Antimicrobial Susceptibility Testing (EUCAST) disk diffusion methods (10), the agar main medium now used for conventional bacterial susceptibility testing is Mueller-Hinton. Sensitest and Iso-Sensitest agars are still used by minor susceptibility testing methods in Australia (11) and the United Kingdom (12). Mueller-Hinton agar has become the de facto standard agar medium in large part because it was selected for disk susceptibility testing when it was first standardized in the United States by Bauer et al. (13). Curiously, the medium was originally developed for the cultivation of *Neisseria* species. A large number of criticisms have been leveled at Mueller-Hinton over the years. Its problems include the possibility of different MIC values in broth versus the agar version, antagonism of tetracyclines, high levels of folate synthesis inhibitor antagonists, variation in performance between manufacturers due to difference in peptone sources, poor support for streptococcal species, and variable growth rates with gram-positive bacteria generally (14). Most of these problems were overcome through the intensive efforts of investigators and manufacturers toward its standardization, including intensive quality control procedures (15) and subsequently the development of "golden pound" reference lots (16). Currently, a new International Standards Organization (ISO) standard is being prepared which permits the calibration of Mueller-Hinton agar and broth based on performance rather than by comparison

to a reference golden pound (17).

Iso-Sensitest agar was a common choice in some European countries until supplemented by EUCAST methods (18). Iso-Sensitest was developed by Oxoid from the original diagnostic sensitivity test agar and then Sensitest agar and is designed to minimize the amount of variable nutrients and maximize the defined components. To a lesser extent, problems have been encountered over the years in the performance of Iso-Sensitest (19). Nevertheless, it remains the basis of the British Society of Antimicrobial Chemotherapy disk diffusion test (12). Paper disk method (PDM) antibiotic sensitivity medium, long recommended (along with Iso-Sensitest) by the Swedish Reference Group on Antibiotics (20), has been withdrawn from the market.

The medium recommended for anaerobe susceptibility testing is Brucella agar or Wilkins-Chalgren (21,22). Less attention has been paid overall to the suitability of different media for anaerobes. Studies by a number of investigators have shown that supplemented Brucella agar supports the growth of a wider range of anaerobes than other media (23–25) and is now recommended as the reference medium by CLSI (22).

There are considerable differences in the formulas of different agars (Table 2.1).

Table 2.1

Formulations of Agar Media Used in Current Susceptibility Testing Methods

	Mueller-Hinton (CLSI, EUCAST)	Iso-Sensitest (BSAC)	Sensitest (CDS)	Wilkins-Chalgren (CA-SFM)	Brucella (CLSI)	GC (CLSI)
Protein source	Dehydrated beef infusion 300 g/L (or similar) Hydrolyzed casein 17.5 g/L	Hydrolyzed casein 11 g/L Peptones 3 g/L	Hydrolyzed casein 11 g/L Peptones 3 g/L	Tryptone 10 g/L Gelatin peptone 10 g/L	Peptone 10 g/L Dehydrated meat extract 5 g/L	Peptones from meat and/or casein source 15 g/L
Sugars		Glucose 2 g/L	Glucose 2 g/L	Glucose 1 g/L	Glucose 10 g/L	
Starch	1.5 g/L	1 g/L	1 g/L			1 g/L
Sodium chloride		3 g/L	3 g/L		5 g/L	5 g/L
Buffers		Sodium acetate 1 g/L Disodium hydrogen phosphate 2 g/L	Buffer salts 3.3 g/L			Dipotassium hydrogen phosphate 4 g/L Potassium dihydrogen phosphate 1 g/L
Calcium	Supplement after autoclaving	Calcium gluconate 0.1 g/L				
Magnesium	Supplement after autoclaving	Magnesium glycerophosphate 0.2 g/L				
Metal salts	Cobaltous sulfate 0.001 g/L Cupric sulfate 0.001 g/L Zinc sulfate 0.001 g/L Ferrous sulfate 0.001 g/L Manganous chloride 0.002 g/L					
Yeast extract				5 g/L		In some formulations
Vitamins		Menadione 0.001 g/L Cyanocobalamin 0.001 g/L		Thiamine 0.00002 g/L	Menadione 0.0005 g/L Haemin 0.005 g/L	
Amino acids		L-Cysteine hydrochloride 0.02 g/L L-Tryptophan 0.02 g/L Pyridoxine 0.003 g/L Pantothenate 0.003 g/L Nicotinamide 0.003 g/L Biotin 0.0003 g/L Thiamine 0.00004 g/L			L-Arginine 1 g/L Sodium pyruvate 1 g/L	
Nucleosides		Adenine 0.01 g/L Guanine 0.01 g/L Xanthine 0.01 g/L Uracil 0.01 g/L	Nucleoside bases 0.02 g/L			
Agar	17 g/L (12–18 g/L)	8 g/L	8 g/L	10 g/L	15 g/L	10 g/L
pH	7.3 ± 0.2	7.4 ± 0.2	7.4 ± 0.02	7.1 ± 0.2	7.5 ± 0.2	7.2 ± 0.2

CLSI, Clinical and Laboratory Standards Institute; EUCAST, European Committee of Antimicrobial Susceptibility Testing; BSAC, British Society for Antimicrobial Chemotherapy; CDS, calibrated dichotomous sensitivity; CA-SFM, Comité de l'Antibiogramme de la Société Française de Microbiologie; GC, gas chromatography.

FACTORS IN AGAR COMPOSITION

Antagonists of Folate Synthesis Inhibitors

Paraaminobenzoic acid (*p*-ABA) is a potent inhibitor of sulfonamides. Concentrations found in certain media such as peptone water and nutrient agar will virtually eliminate sulfonamide activity (26). Susceptibility testing agar used in any of the current published methods has minimal concentrations of *p*-ABA.

Thymidine and thymine in sufficient concentrations antagonize the dihydrofolate reductase (DHFR) inhibitors such as trimethoprim, increasing MICs and reducing zone diameters in agar diffusion tests. This nucleoside and its pyrimidine base possibly act by competing for the target enzyme. Thymidine is by far the more potent of the two, and methods to reduce or eliminate its presence in agar will restore DHFR inhibitor activity. Indeed, one common method is to use lysed horse blood, which is rich in the enzyme thymidine phosphorylase, which converts thymidine to thymine and 2-deoxyribose-1-phosphate. Further, most bacteria, except *Enterococcus faecalis*, cannot utilize thymine as a substrate (26). Some bacterial strains require thymidine for growth and will grow poorly or not at all on susceptibility testing agar. Because such strains are naturally DHFR inhibitor resistant, no problems of testing occur when thymidine is added back into the medium.

Although Mueller-Hinton agar is purportedly low in thymidine and thymine, occasionally, problems can arise. CLSI has developed a quality control procedure to test for low thymidine content using either of two American Type Culture Collection (ATCC) strains of *E. faecalis* and trimethoprim-sulfamethoxazole disks (27). This procedure is now embraced in the new ISO standard to Mueller-Hinton lot acceptance criteria (17).

Calcium, Magnesium, Zinc, and Manganese

The principal problem with Mueller-Hinton over the years has been the variability in the concentrations of divalent cations, especially calcium (Ca^{++}) and magnesium (Mg^{++}), which can have significant effects on the activity of aminoglycosides and some other antimicrobials, particularly against *Pseudomonas aeruginosa* (28,29). For the broth, this has now been overcome during the manufacturing process, and the concentrations are adjusted to within an acceptable range specified by ISO 16782 (17). However, the addition of agar to Mueller-Hinton will alter the cation concentration in unpredictable ways, such that for some newer antimicrobial agents heavily influenced by cation concentration, such as Ca^{++} with daptomycin (30) and manganese (Mn^{++}) with tigecycline (31,32), it has not been possible to develop agar dilution or diffusion standards. The great variability in cation concentrations with different brands of Mueller-Hinton agar has recently been highlighted (31,33).

The divalent cations of Ca^{++} and Mg^{++} are well-known antagonists of aminoglycosides. The antagonism is complex and cannot simply be accounted for by the concentrations of the cations themselves (34). It appears to be affected to a large extent by other constituents such as sodium chloride and phosphate. The effects are most obvious when testing *P. aeruginosa* (35–37). The variability of concentrations in Mueller-Hinton agar in the past has been one reason that certain European countries have favored Iso-Sensitest agar, where the divalent cation concentrations are defined in the formulation. Ostensibly, agar itself is processed to remove free cations and anions (14), but recent studies have demonstrated that there can still be a wide range of calcium and magnesium concentrations in different batches of Mueller-Hinton agar (30,31,33). In contrast to Mueller-Hinton broth, the concentration of divalent cations in Mueller-Hinton is not stipulated in the ISO 16782 standard. However, other susceptibility test media have not been subject to control of their divalent cations, and the reproducibility of aminoglycoside results with different lots has not been examined.

The concentration of Ca^{++} is critical to the interpretation of daptomycin susceptibility (30). Daptomycin activity varies greatly with Ca^{++} concentration, and specified concentrations are required in the media (usually those seen physiologically). For this reason, daptomycin has not yet been completely standardized for tests in agar, and supplementation is required for broth testing (30). Cation concentrations are also known to affect the activity of the polymyxins in susceptibility tests at least for *P. aeruginosa* and *Acinetobacter baumannii* (33,35). Calcium and magnesium concentrations can also affect the action of tetracycline against *Pseudomonas* species, although this is of little importance because tetracyclines are not considered clinically active against these species.

The concentrations of another cation, zinc (Zn^{++}), in Mueller-Hinton (38–40) agar and Iso-Sensitest agar (41) has an impact on the activity of imipenem and possibly other carbapenems, at least for common nonfermentative gram-negative. Again, the concentrations of Zn^{++} can be quite variable in different brands of Mueller-Hinton agar (32,33).

A summary of two recent publications that have examined cation concentrations in Mueller-Hinton agar is presented in Table 2.2.

Table 2.2

Variations in Cation Concentration in Commercial Formulations of Mueller-Hinton Agar

		Cation Concentration									
Reference	**Manufacturer**	**Ca**	**Mg**	**Zn**	**Fe**	**Ni**	**Cd**	**Mn**	**Cu**	**Pb**	**Hg**
Fernández-Mazarrasa et al. (31)	Merck	12.2	8.2	0.18	0.5	0.08	0.01	11.5	0.08	0.01	bld
	Difco	13.5	7.8	0.38	0.3	0.08	0.01	0.05	bld	0.02	bld
	Oxoid	13.8	16.8	0.14	0.6	0.08	0.01	0.05	bld	0.02	bld
Girardello et al. (33)	Merck	7.5	6.2	bld	bld			19.3			
	Difco	16.1	4.9	1.1	bld			bld			
	Oxoid	20.9	13.3	bld	bld			bld			
	Himedia	25.3	31.2	bld	bld			bld			

Ca, calcium; Mg, magnesium; Zn, zinc; Fe, iron; Ni, nickel; Cd, cadmium; Mn, manganese; Cu, copper; Pb, lead; Hg, mercury; bld, below limit of detection.

Sodium Chloride

Less well known is the effect of sodium chloride concentration on the activity of aminoglycosides (42,43). As summarized by Waterworth (34), variations in NaCl can have quite significant effects: an increase in concentration from 22 to 174 mM can increase the MIC of gentamicin by as much as 32-fold. NaCl is not part of the Mueller-Hinton formulation, but the manufacture to a reference standard at least generates consistent results. NaCl is part of the Iso-Sensitest formulation, but viable amounts could also come from the hydrolyzed casein and peptones.

pH

Major variation in the pH of the medium can result in major changes in the activity of aminoglycosides, macrolides, and tetracyclines. Aminoglycoside activity is substantially increased in alkaline conditions and substantially inhibited in acidic conditions. Similar effects are observed with macrolides. Susceptibility testing agars are manufactured to performance standards of pH, and in some testing methods, such as those of CLSI, it is recommended to confirm the pH in the cooled, prepoured state or with a surface pH meter after pouring, once the agar has been prepared from the dried powder in the routine laboratory.

The pH effect does become significant, however, when agar plates are incubated in increased concentrations of CO_2; such is recommended for streptococci and *Haemophilus* species in most methods. Rosenblatt and Schoenknecht (44) have shown an increase in pH from 7.4 to 8.4 over a period of 24 hours when Mueller-Hinton blood agar plates are incubated in 5% to 7% CO_2 in air. Carbon dioxide is absorbed onto the surface during incubation, some of which will be converted to carbonic acid initially and then carbonate ions, first decreasing and later increasing the pH at the surface (44). Acidity is known to reduce the activity of macrolides in particular (including the azalides and ketolides) (45–55) and of aminoglycosides to some extent (56,57) while increasing the activity of tetracyclines (2). In agar-based tests, this effect will result in higher MICs and smaller zones for macrolides and aminoglycosides, as the pH at the surface will be more acidic at the critical time.

Additives

Some bacterial species require the addition of specific nutrients to ensure adequate growth. The most common of these is blood, usually sheep or horse blood, at a concentration of 5%. When testing sulfonamides, horse blood is preferred, as it is low in sulfonamide antagonists.

Specific reagent additives are required for certain fastidious species or for nonfastidious species against certain antimicrobial agents, as described in Table 2.3.

Table 2.3

Specific Additives to Agars Used for Susceptibility Testing

Method	Medium	Species	Antimicrobial Agent(s)	Additive(s)
CLSI agar dilution and disk diffusion	Mueller-Hinton	*Staphylococcus* spp.	Oxacillin, nafcillin, methicillin	2% NaCl
		All relevant	Fosfomycin	25 mg/L Glucose-6-phosphate
		Streptococcus spp. *Neisseria meningitidis* *Campylobacter jejuni/coli* *Pasteurella* spp *Mannheimia haemolytica*	All relevant	5% Sheep blood
		Helicobacter pylori	All relevant	5% Aged sheep blood (≥2 weeks old)
		Haemophilus spp	All relevant	15 mg/L β-NAD 15 mg/L Bovine or porcine hematin 5 g/L Yeast extract ±0.2 IU Thymidine phosphorylase (if testing folate antagonists) (*Haemophilus* test medium)
		Histophilus somni *Actinobacillus pleuropneumoniae*		10 g/L Hemoglobin 1% Defined growth supplement ("Chocolate Mueller-Hinton")
	GC base	*Neisseria gonorrhoeae*	All relevant	1% Defined growth supplement
	Brucella	Anaerobes	All relevant	5% Laked sheep blood 5 mg/L Hemin 1 mg/L Vitamin K_1
EUCAST disk diffusion	Mueller-Hinton	*Streptococcus* spp *Haemophilus* spp *Moraxella catarrhalis* *Listeria monocytogenes*	All relevant	5% Horse blood 20 mg/L β-NAD (Mueller-Hinton-F)
BSAC disk diffusion	Iso-Sensitest	*Streptococcus pneumoniae* β-Hemolytic *Streptococcus* spp *Moraxella catarrhalis* *Neisseria gonorrhoeae* *Neisseria meningitidis* *Campylobacter* spp.	All relevant	5% Horse blood
		α-Hemolytic *Streptococcus* spp *Haemophilus* spp *Pasteurella multocida* *Bacteroides fragilis* *Bacteroides thetaiotaomicron* *Clostridium* spp *Coryneform bacteria*	All relevant	5% Horse blood 20 mg/L β-NAD

Table 2.3 (Continued)

Specific Additives to Agars Used for Susceptibility Testing

Method	Medium	Species	Antimicrobial Agent(s)	Additive(s)
CDS disk diffusion	Sensitest	*Corynebacterium* spp *Enterococcus* spp *Listeria* spp *Streptococcus* spp *Erysipelothrix rhusiopathiae* *Moraxella catarrhalis* *Campylobacter* spp *Neisseria meningitidis* *Pasteurella* spp	All relevant	5% Horse blood
	Mueller-Hinton	*Haemophilus* spp	All relevant	15 mg/L β-NAD 15 mg/L Bovine or porcine hematin 5 g/L Yeast extract
	Columbia	*Helicobacter pylori* *Neisseria gonorrhoeae*	All relevant	8% "Chocolatized" horse blood
	Brucella	Anaerobes		5% Horse blood 5 mg/L Hemin 1 mg/L Vitamin K_1

CLSI, Clinical and Laboratory Standards Institute; NAD, nicotinamide adenine dinucleotide; GC, gas chromatography; EUCAST, European Committee of Antimicrobial Susceptibility Testing; BSAC, British Society for Antimicrobial Chemotherapy; CDS, calibrated dichotomous sensitivity.

Defined Growth Supplement

One important additive, noted here and in CLSI documents as *defined growth supplement*, is a complex mixture of vitamins, cofactors, and other nutrient substances. The two most recognizable and important brands are IsoVitaleX (Becton, Dickinson and Company, Sparks, MD) and Vitox (Thermo Scientific, West Palm Beach, FL). These products contain 1.1 g L-cystine, 0.03 g guanine, 3 mg thiamine HCl, 13 mg *p*-ABA, 0.001 to 0.012 g vitamin B_{12}, 0.1 g cocarboxylase, 0.25 g nicotinamide adenine dinucleotide (NAD), 1 g adenine, 10 g L-glutamine, 100 g glucose, 0.02 g ferric nitrate, and 25.9 mg cysteine HCl per liter of water. It is most commonly used at a 1% concentration to ensure the growth of the fastidious organisms, especially *Neisseria gonorrhoeae*.

PARANITROPHENYLGLYCEROL AND OTHER ANTISWARMING AGENTS

Paranitrophenylglycerol (PNPG) has been used among other techniques to prevent the swarming of *Proteus mirabilis* and *Proteus vulgaris* across agar surfaces. This is mostly a problem for agar dilution testing when multiple strains including strains of these two species are being tested on a single series of plates. As a result of the swarming, spots close to the *Proteus* species can be difficult or impossible to read. However, PNPG has been shown to affect the MIC results of a number of bacterial species and antimicrobials, especially *Pseudomonas aeruginosa* (58,59).

A second antiswarming agent, Dispersol LN, has also been evaluated and shown to affect the activity of some antimicrobials (60). The addition of PNPG or another antiswarming agent is no longer recommended routinely in any method and should only be used if there are data to show that the agent does not interfere with the MICs of that organism–antimicrobial combination. Another natural chemical, 10′(*Z*),13′(*E*)-heptadecadienylhydroquinone, has been shown to increase susceptibility of *P. mirabilis* to polymyxin B (61).

Higher concentrations of agar (e.g., 2%) can be used to inhibit swarming. None of the three options—use of PNPG, use of Dispersol LN, or increasing the concentration of agar—is now recommended.

Strains with Special Growth Requirements

Special problems arise with strains or species with unusual growth requirements. The so-called nutritionally variant "streptococci" *Abiotrophia defectiva* (previously *Streptococcus defectivus*) and *Granulicatella adiacens* (previously *Streptococcus adjacens*, *Abiotrophia adiacens*) require pyridoxal for growth. Media can be supplemented with pyridoxal (0.001%) and lysed horse blood to ensure growth of the strains (63–65) and allow interpretation of MICs at least (no breakpoints have been determined).

Occasionally, mutant strains of *Staphylococcus aureus* will depend on thiamine or menadione (vitamin K_3) for growth. These often exhibit small colonies on primary isolation. The addition of thiamine (2 mg/L) and menadione (0.5 mg/L) to susceptibility testing media will allow susceptibility (MIC) tests to be performed (66).

Strains of *Escherichia coli* and other gram-negatives dependent on thymine for growth are sometimes encountered. They can be selected for during treatment with folate synthesis inhibitors (67). Strains of Enterobacteriaceae dependent for growth on thymidine, cysteine, or glutamine may be tested by adding the appropriate nutrient to the basal medium (7), although experience with this is limited due to the infrequency with which these strains are isolated.

AGAR DILUTION SUSCEPTIBILITY TESTING

Agar dilution susceptibility testing is the solid equivalent of broth dilution susceptibility testing, either in macro- or microbroth format. One advantage it offers over broth-based methods is that it allows the simultaneous testing of a large number of strains on a single agar plate, for example, 32 on a 90-mm plate using a Steers-Foltz or similar replicator (Fig. 2.1). It is therefore well suited to the rapid evaluation of new compounds or for large-scale centralized surveillance programs. The comparative disadvantages of this method are that it includes an additional variable (agar) in the medium and that, once prepared, the plates have a limited shelf life owing to degradation of the antimicrobial.

Figure 2.1 ■ Replicator for agar dilution. (See Color Plate in the front of the book.)

Most important, although agar dilution susceptibility testing, such as that described by CLSI (62) and EUCAST (18), has traditionally been accepted as equivalent to broth microdilution, it is not the international (ISO) reference standard, and users of agar dilution need to be aware that there may be differences from the ISO standard for some antimicrobial agent–microorganism combinations, particularly in the light of uncontrolled cation concentrations in Mueller-Hinton agar versus Mueller-Hinton broth as specified for susceptibility testing. In the future, it will be necessary to establish equivalence to the ISO standard for new antimicrobial agents as they are developed. Methods for establishing equivalence have been described (68).

The two major published agar dilution susceptibility testing methods for common human pathogens (18,62) are essentially equivalent. In the usual approach to determining MICs on agar, the antimicrobial is incorporated into molten agar over a series of twofold dilutions, gently rotated to ensure even distribution of the antimicrobial, and then poured into Petri plates. The major elements of the test are described in the following sections.

Antimicrobial Powders

Antimicrobial powders should be obtained as "pure substance" from the manufacturer or from a reputable chemical supplier (e.g., Sigma-Aldrich Corporation). It is not appropriate to use powders found in vials for parenteral drug administration, as these may contain preservatives, surfactants, fillers, or other substances that could interfere with the antimicrobial activity, and their contents may vary legally by as much as 10% above or below the label amount. Each powder should come with an expiration date and an indication of its potency and (sometimes) water content. It is vital that all these data be taken into account before preparation of stock solutions. Data should also be available from the manufacturer or another reliable source on choice of solvent and solubility before preparing stock solutions. One easily accessible source is Table 5A in CLSI's M100 document, which is updated yearly (69).

Powders should be stored as recommended by the manufacturer. If no instructions for storage are available, then store powders at −20°C in a desiccator, preferably under vacuum (69). This will ensure the product retains its potency for the maximum time.

Choice of Dilution Range

Before preparing stock solutions, it is essential that an appropriate dilution range be chosen. Suggested ranges for different species or bacterial types have been published (21). A full range is generally 10 to 12 doubling dilutions. Doubling dilutions are appropriate because they provide the narrowest integer series on a logarithmic scale, and MICs for a single bug–drug combination are log-normally distributed in the wild type, that is, in the absence of a resistance mechanism. Although any series could be used, the most widely accepted doubling dilution series is that of base 2. This implies that preferably the highest concentration (the concentration at the start) should be a power of 2 (e.g., $2^7 = 128$). Following this pattern will allow comparison with the majority of published data and MICs for published quality control strains.

Preparation of Stock Solutions

Stock solutions should be prepared by weighing out the *exact amount* of powder using a balance designed for milligram amounts. Ideally, amounts less than 100 mg should not be weighed out. Alternatively, an approximate amount can be weighed out, and using the following formula, the *exact volume* can be added. The amount weighed is calculated using the formula

$$W = \frac{V \times C}{P}$$

where W = weighted amount (μg), V = volume of stock solution required (mL), C = concentration of solution required (μg/mL), and P = potency in μg/mg. Usually, a potency value is provided. If not, it will need to be calculated from the values provided in the certificate of analysis. These values are purity (as measured, e.g., by high-performance liquid chromatography [HPLC]), water content (as measured, e.g., by Karl Fischer analysis), and active fraction, which will be lower for salts than for free acids or bases.

$$\text{Potency} = \text{Purity} \times \text{Active fraction} \times (1 - \text{Water content [\%]})$$

The choice of final concentration will depend on whether the antimicrobial is being used immediately or whether it is intended for aliquoting and storage. Stock solutions for storage are best prepared as a 10-fold concentration of the highest dilution being used. Concentration choices will also be determined by the solubility of the antimicrobial. Stock solutions should be stored frozen at −20°C or lower and only thawed once before use; any unused thawed stock should be discarded.

Some antimicrobial agents require special solvents to achieve the high concentrations required in stock solutions. A comprehensive list of appropriate solvents and diluents is provided in reference 70 and is kept updated on a yearly basis as new antimicrobial agents in clinical

development are added.

Preparation of Dilution Range

In order to avoid compounding minor errors in pipetting that would occur if low volumes were used or with repeated dilution, dispensing and dilution schemes such as those recommended by CLSI (69), the British Society for Antimicrobial Chemotherapy (BSAC) (21), and EUCAST (18) should be followed. These are adaptations of the original dilution scheme proposed by Ericsson and Sherris (71). An antimicrobial-free control plate should be added to any dilution series for quality control (i.e., to ensure that the selected strains are indeed viable).

Agar Selection

The description of each method identifies which agar media to use for particular bacterial groups or species. As all subsequent interpretations depend on data generated from these media, the nominated media for each method must be used. The ranges of media specified for each method are listed in Table 2.1.

Preparation of Agar Plates

Agars are generally prepared from a dehydrated base following the manufacturer's instructions with regard to amounts of base, water, and autoclaving. After autoclaving, bottles should be cooled to 45°C to 50°C by placing them in a water bath at this temperature. When the agar has reached this temperature, antimicrobial dilutions and supplements—and blood if required—are added aseptically. An additional plate should be prepared without the incorporation of antimicrobials as a plate to ensure that there is adequate growth of test and control strains on the medium.

Adequate mixing of the antibiotic in the molten agar is essential, and this is best achieved by mixing each antibiotic solution in a small decanted volume of molten agar and then mixing the result with the bottle of molten agar. If the antibiotic solution is added directly to the whole bottle of molten agar, the solution should be warmed to avoid small portions of agar solidifying around the solution as it is added. The antimicrobial solution is mixed into the molten agar by gentle swirling and inverting the bottle rather than by shaking so that frothing is avoided. Plates should be poured onto a level surface as soon as practical after mixing.

The pH of each batch of agar should be measured after preparation. An aliquot of molten agar can be poured into a beaker or cup over a suitably designed pH electrode and allowed to gel and reach room temperature. Alternatively, a surface electrode can be placed on an aliquot of agar poured into a Petri dish and allowed to reach room temperature. It is also possible to macerate a sufficient amount of set agar and submerge the tip of an electrode.

Agar plates are then poured from the cooled molten agar as soon as possible after mixing in order to minimize any impact on the antimicrobial concentration. There is no specified depth, but 20 mL of molten agar in a 90-mm plate is considered adequate. It is advisable to dry the plates, for instance, in a fan-assisted drying cabinet for 10 minutes (21) or in a 35°C to 37°C incubator inverted with the lids off (72), as moisture buildup is common if covers are placed over cooling agar in Petri dishes.

Preparation of Inoculum

Most standards offer the choice of two methods for the preparation of inocula: the "growth" method and the "direct" method. The direct method is often preferred for inocula prepared from fastidious organisms, as growth of these organisms in broth can be a little unpredictable. The solution for final suspension varies somewhat between standards and sometimes between groups or species being tested but is generally either 0.85% to 0.9% NaCl, the same type of broth used in the agar, or phosphate-buffered saline (PBS). For reference work, inocula should be prepared from subcultures in order to ensure purity.

Calibration of Turbidity

Inocula are always calibrated to a turbidity standard, usually a McFarland turbidity standard, which employs particulate suspensions of barium sulfate. McFarland 0.5 is the most common choice and can be prepared as described in various texts (62,72). Briefly, it is a 0.5-mL aliquot of 1.175% w/v $BaCl_2 \cdot 2H_2O$ added to 99.5 mL of 1% w/v H_2SO_4 (62). Alternative turbidity standards made from other materials such as latex particles but optically equivalent to 0.5 McFarland are available commercially (e.g., Remel Inc, Lenexa, KS). Normally, a visual comparison is made with the turbidity standard. It is important that this be done in good lighting and against a card with a white background and a contrasting black line. More recently, there has been a move to the use of a nephelometer or photometer to achieve this calibration. For instance, at a wavelength of 550 nm, a 5-mL glass tube with 2 mL of inoculum suspension with an optical density of 0.1 to 0.12 approximates a 0.5 McFarland barium sulfate standard (72). Equally, at 625 nm and a light path of 1 cm, the standard has an optical density of 0.08 to 0.10 (62).

Barium sulfate standards are affected by light and heat, and hence they should be stored between uses in a dark place at room temperature. Their turbidity should be checked in a nephelometer or photometer monthly. They also require vigorous agitation at each use.

Growth Method

Usually, two to five morphologically similar colonies are picked from a primary or subculture plate. The wire loop is used to touch each colony, and it is then immersed in about 5 mL of the recommended broth (e.g., tryptic soy broth in the CLSI method). The broth is incubated at 35°C until it equals or exceeds the correct turbidity, generally 2 to 6 hours for rapidly growing pathogens. The broth is then diluted with a suitable sterile fluid such as broth, saline, or PBS according to the instructions until 0.5 McFarland is reached.

Direct Colony Suspension Method

In this method, two to five colonies are touched or picked up and suspended directly in the fluid of choice. Clearly, the turbidity should be adjusted subsequently as for the growth method. This method is acceptable in almost all situations. Hence, it remains an option. The choice of growth versus

direct method depends on laboratory workflow. Almost all standards prefer the direct suspension method for fastidious organisms such as *Haemophilus* species and *N. gonorrhoeae*.

Plate Inoculation

In almost all circumstances, it is preferable to use a replicator apparatus to inoculate the agar plates, such as a Steers replicator (see Fig. 2.1). These are expensive items but last indefinitely. They generally come with 32 to 36 pins to deliver this number of strains to a 90-mm agar plate. The size of the pins is critical to decisions about how or whether the inoculum to be used for various species is to be further diluted. Prongs of 3 mm deliver approximately 2 μL (range 1 to 3 μL) to the plate surface; prongs of 2.5 mm generally deliver around 1 μL (18), while prongs of 1 mm deliver around 10-fold less (0.1 to 0.2 μL) (73). Hence, some standards recommend dilution for some organism groups or species when using the 3-mm pins.

There is some variation between standards regarding whether further dilution of the standardized inoculum prepared as outlined earlier should be performed and how it should be performed. The commonly stated intention is that each spot on the plate should contain around 10^4 colony-forming units (CFUs). However, in preparing standardized inocula, there are considerable differences between species in the number of CFU per milliliter for a given turbidity (74).

Incubation

Temperature and Duration

Most organisms should be incubated at 35°C to 37°C. An incubator set at 36°C can generally operate within this range. The CLSI method specifies 35°C ± 2°C for all bacteria. The duration of incubation depends on the species. For rapidly growing species such as the Enterobacteriaceae, the glucose-nonfermenting gram-negative bacilli, *Enterococcus* species, and *Staphylococcus* species, overnight incubation for a minimum of 16 hours is needed. Longer incubation is recommended for fastidious species (18 to 20 hours plus), for *Staphylococcus* species when testing against antistaphylococcal penicillins (24 hours), for *Campylobacter* species (24 to 48 hours depending on the standard and incubation temperature), for *Helicobacter pylori* (CLSI 3 days), and for anaerobes (42 to 48 hours).

Atmosphere

Enrichment of air with CO_2 is recommended in most standards for a variety of fastidious species, including *Streptococcus pneumoniae*, *Haemophilus influenzae*, sometimes other *Streptococcus* species, and *N. gonorrhoeae*. The concentration is usually 5%, although some standards tolerate ranges of 4% to 6%. The effect on the activity of certain drugs under these circumstances was discussed earlier.

Campylobacter species and *H. pylori* demand incubation in a microaerophilic atmosphere, identical to that recommended for primary isolation. Commercial systems are available for generating the required microaerophilic environment.

Anaerobic bacteria should obviously be incubated in the standard anaerobic environment used for primary isolation of anaerobes. Either an anaerobic chamber or jar is acceptable.

Plate Stacking

The effect of stacking plates on the incubation temperature is not widely recognized. After placement in the incubator, plates in the middle of a stack will take longer to reach the desired incubation temperature than plates at the top and bottom (75,76). This is important because the incubation temperature can have a profound effect on the generation time of bacteria, which in turn will affect the end point determination. In general, no more than five plates should be used in a stack. Even then, it can take up to 4 hours for the center plate to reach the incubator temperature (76).

Reading

Plates should be read with optimum lighting, preferably on a dark, nonreflecting surface. For instance, in Figure 2.2, growth has obviously occurred at some spots but not at others. The MIC is taken as the first concentration at which no growth occurs. Most standards recommend that the appearance of one or two colonies or a faint haze can be ignored. However, if there are one or two colonies at a number of concentrations rather than a single one above the putative MIC, then the MIC is that concentration at which no colonies are seen. If this is the case, the purity of the strain should be checked, as it may be an indication of contamination. The only significant exception to these reading rules occurs in the reading of the end points for folate synthesis inhibitors, where growth can diminish gradually over a range of concentrations. CLSI recommends that the end point be read as that concentration resulting in 80% inhibition of growth (62). Other bacteriostatic drugs, such as chloramphenicol, clindamycin, tetracycline, and linezolid, can exhibit the same phenomenon to a lesser degree.

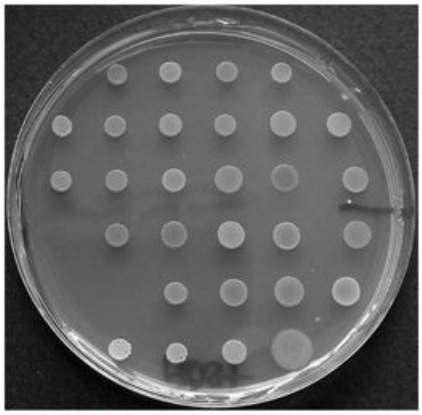

Figure 2.2 ■ Agar dilution plate after 24 hours of incubation. The medium is Mueller-Hinton agar. The strains are of *Staphylococcus aureus*. The last spot at bottom right is a control. Three strains have not grown at this concentration. (See Color Plate in the front of the book.)

Quality Control

Quality control procedures in the performance of agar dilution are designed to ensure the reproducibility of the test and to confirm the performance of the reagents and the persons conducting the test. Most importantly, it is designed to detect errors of concentration or dilution of the antimicrobials, a not infrequent hazard in agar dilution testing. It is assumed that the laboratory is using reagents and materials from trusted suppliers who undertake quality control checks during manufacture. Using a trusted supplier does not prevent problems that may develop during shipping and handling, and hence, there is a need to have a quality control system at the laboratory level.

Quality Control Strains

The main component of quality control is the testing of reference quality control strains. The number tested varies between standards and with the type of strains being tested. Wherever possible, it is desirable to test a quality control strain of the same family, genus, and species as the strains under examination. The following ATCC reference strains have become almost universal as quality control strains for testing rapidly growing aerobic bacteria: *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, *P. aeruginosa* ATCC 27853, and *Enterococcus faecalis* ATCC 29212. Other strains recommended by different standards include *Escherichia coli* ATCC 35218 (CLSI for β-lactamase inhibitor combinations), *Staphylococcus aureus* Collection de l'Institut Pasteur (CIP) 6525 (Comité de l'Antibiogramme de la Société Française de Microbiologie [CA-SFM] for methicillin and oxacillin), *Haemophilus influenzae* ATCC 49217 (CLSI and BSAC), *Haemophilus influenzae* ATCC 49766 (CLSI), *Haemophilus influenzae* National Collection of Type Cultures (NCTC) 8468 (EUCAST), *N. gonorrhoeae* ATCC 49226 (CLSI and BSAC), *Streptococcus pneumoniae* ATCC 49619 (CLSI and BSAC), *Helicobacter pylori* ATCC 43504 (CLSI), *Campylobacter jejuni* ATCC 33560 (CLSI), *Bacteroides fragilis* ATCC 25285 (CLSI), *B. fragilis* NCTC 9343 (BSAC), *Bacteroides thetaiotaomicron* ATCC 29741 (CLSI), *Eubacterium lentum* ATCC 43005 (CLSI), and *Clostridium difficile* ATCC 700057. The CLSI also has special control strains for veterinary testing: *Histophilus somni* ATCC 700025 and *Actinobacillus pleuropneumoniae* ATCC 27090.

The selection of quality control strains involves a compromise between the objective of getting strains with MICs close to the center of a dilution series of a range of antimicrobials (62) and the number of strains that would be required to achieve this objective. The strains commonly recommended have also been selected for their stability over long periods of time and repeated subculture. There is nothing to prevent a laboratory from developing and using its own quality control strains.

Storage of Quality Control Strains

Stock cultures of the quality control strains should be maintained in the freezer below −20°C (ideally at −70°C) in a suitable stabilizer such as fetal calf serum, glycerol broth, or skim milk or should be freeze-dried. Some workers recommend that two sets of stock cultures be stored: one set to create working cultures and the other as emergency backup. This will guarantee that the laboratory always has unaltered quality control strains. Working cultures should be stored on agar slants at 2°C to 8°C and subcultured each week for no more than 3 successive weeks. New working cultures are generated each month from frozen or freeze-dried stock and subcultured twice before use.

Frequency of Testing and Corrective Action

Each standard provides MIC quality control ranges for these strains against some or all of the antimicrobials of interest. The recommended quality control strains are included in each test run. When these strains fall within the quality control range, the test run is valid. When one or more results are out of this range, the test run must be considered invalid and be rerun.

Use in Routine Susceptibility Testing

Agar dilution can be adapted to routine susceptibility testing. Essentially, it is a truncated method that incorporates one or two selected concentrations of antimicrobial, usually at breakpoint values (so-called breakpoint susceptibility testing). It has been advocated as an option for routine testing by some authorities in the past (77) but is slowly being supplanted by other methods. The advantages and disadvantages of this method have been described in detail by BSAC (77). When testing multiple strains of gram-negative bacteria that may include *P. mirabilis* and *P. vulgaris*, it is necessary to include an antiswarming agent, which, as discussed earlier, can affect results. The method offers specific challenges in

terms of quality control because the limited number of concentrations will only detect the most gross of preparation errors in standard quality control organisms and will often fail to detect the most common error—a 10-fold error in antibiotic dilutions. To overcome this, there are a number of options available: (a) assaying antibiotic dilutions prepared from stock solutions, (b) assaying agar plugs removed from a poured agar plate (78), and (c) assaying paper disks applied for a fixed interval to the surface of a poured agar plate (79). McDermott et al. (79) provided a detailed analysis of two of these methods and recommended methods with high precision.

DISK DIFFUSION SUSCEPTIBILITY TESTING

Disk diffusion susceptibility testing has a long history, having evolved out of antibiotic diffusion from wells used for drug assay and susceptibility testing. The method in its various forms still has wide popularity owing to its ease of use and low cost compared with other methods. It has spawned many variants around the world. Unlike in dilution methods, an MIC value is not generated. Instead, in the development of the test, zone diameters must be compared with the MIC values of the same strains in order to determine which zone diameters predict which MIC values and hence which category of susceptibility.

Theoretical Aspects

All disk diffusion methods are based on the diffusion through agar of drug released from an impregnated disk. There are a large number of variables affecting this diffusion. Important features of antibiotic diffusion were worked out by Cooper and others in the 1950s (75,76,80–83). They have been clearly explained by Barry (3,84), who detailed the dynamics of zone formation and the "critical concentration," "critical time," and "critical population."

When an antibiotic is placed in a well cut into the agar or in a disk applied to the agar surface, the drug commences diffusion immediately and diffuses in a decreasing gradient of concentration from the edge of the well or disk. Over a number of hours, the height of this gradient deceases from very steep initially to quite shallow as the drug continues to diffuse (85). In disk susceptibility testing, disks are applied after the surface has been inoculated with bacteria. The formation of the zone edge is thus a contest between the diffusion of the drug and the growth rate of the bacterial inoculum, including any initial lag phase after incubation commences. The critical concentration is the concentration just capable of inhibiting growth, and it is also that concentration found at the zone edge at the critical time. It is similar but not identical to the MIC as measured by dilution methods. The critical time is the time it takes for the critical concentration to be reached at what ultimately becomes the zone edge. It is generally around 3 to 4 hours under standard test conditions. The critical population is the number of bacterial cells found at the critical time at the ultimate zone edge. The relationships between these parameters are as follows:

$$C_c = \frac{M}{4\pi D T_0 h} e^{\frac{r^2}{4DT_0}}$$

$$T_0 = L + G \log_2 \left(\frac{N'}{N_0}\right)$$

where C_C = critical concentration, M = disk content, T_0 = critical time, D = diffusion coefficient of drug, h = depth of agar, r = zone radius, L = lag time, G = generation time, N'^2 = critical population at the critical time, and N_0 = number of viable cells at beginning of incubation. Although the following description is inaccurate, it is useful to simplify these relationships by imagining the zone of inhibition as a "cylinder" in which the drug is evenly distributed. The concentration of drug in this cylinder is thus the disk content (M) divided by the volume of the cylinder, namely $pd^2h/4$, where d is the zone diameter and h is the depth of the agar and therefore $d = \sqrt{4M/\pi hC}$. Thus, the zone diameter increases in proportion to the square root of the disk content and decreases in proportion to the square root of the agar depth. This defines the essential relationships between zone size, disk content, and agar depth and how variations in these affect zone diameters.

Disk Production

Disks for almost all antimicrobials can be obtained commercially. Even drugs that are still under development are likely to have disks available for use in the laboratory and clinical development programs. From time to time, it may be useful for a laboratory to manufacture its own disks. Strict standards must be adhered to if this is done. The same stipulations in drug sourcing and preparation of stocks as have been described in the section on agar dilution apply. Paper disks should be obtained that adhere to the same standards that apply to commercial manufacturers. In the United States, the standard is 740-E (Schleicher and Schuell, Keene, NH), and the paper used should be 30 ± 4 mg/cm^2 (86).

Solvents used in disk manufacture are described in a previous edition of this book (86).

Factors Influencing Zone Diameters

A large range of factors can influence the zone sizes produced. The most important are the disk content (also called *potency*, *mass*, or *strength*, namely, the amount of drug in the disk), the disk size, the diffusion characteristics of the drug, the depth of the agar, the growth rate of the bacterium (including the initial lag phase), the density of the inoculum, and the activity of the drug against the strain being tested. Other factors such as medium composition, pH, and the effect of additives are dealt with at the beginning of this chapter.

Disk Content

The amount of drug impregnated into the disk is somewhat arbitrary. Amounts are chosen that are likely to produce zones of moderate size (15 to 35 mm) under normal conditions. Different disk methods have often selected different disk contents based on early experience with the antibiotic during development and precedents set within antibiotic classes.

Disk contents will be subject to variation during manufacture, and regulators such as the U.S. Food and Drug Administration (FDA) have set tolerances on the true amount in the disk in the range of 90% to 125% of the label. Such small errors will have a small effect on the zone diameter

because it is proportional to the square root of the disk content, which means that the possible variation in the diameter ranges from about −5% to +12%.

The difference in zone diameters with different disk contents has been exploited to determine critical concentrations (84). When three or more disks with a range of antibiotic contents are used, the resulting zone diameters, when squared, are directly proportional to the logarithms of the disk contents. The results can be plotted using linear regression, extrapolation of which to no zone yields the critical concentrations. These values will often be good approximations of the MICs as measured by other methods (3).

Disk Size

The extent of drug diffusion will obviously be affected by the width of the disk. Paper disks are now almost universally manufactured to be 6 mm wide. Further, the nature of the paper is subject to regulation, as different varieties of paper have been shown to affect the release characteristics of antibiotics (86). One disk method, that of Neo-Sensitabs produced by the Danish company Rosco Diagnostica A/S, Taastrup, Denmark (87,88), uses 9-mm disks made from hardened inert "chalk-like" substances. These larger disks result in larger zones for the same disk content.

Diffusion Characteristics of the Drug

The two crucial properties of the drug molecules are size and charge. In general, larger molecules diffuse more slowly, and the zones formed as a consequence are smaller. Large molecules demonstrating this include the glycopeptides such as vancomycin and teicoplanin (89). Strongly cationic molecules such as the polymyxins polymyxin B and colistin will also be inhibited in their diffusion owing to interaction with acid or sulfate groups on the agar polymer. Aminoglycosides are cationic to a lesser extent, and their diffusion is reduced slightly as a result (2).

Agar Depth

As noted, agar depth will naturally alter the size of the inhibition zone. Most methods have settled on a depth of 4 mm or a similar amount. This represents a balance between a smaller depth, which is likely to generate a reasonable zone size, and a larger depth, which is designed to reduce the zone size variation due to small variations in depth. A number of studies have demonstrated greater plate-to-plate variation in zone diameter when the agar depth is less than 3 mm (89a,89b).

Time between Inoculation and Disk Placement

The period of delay between the inoculation of the agar surface and the placement of the disks prior to incubation will have a significant effect on the ultimate zone diameter. This is a direct result of the concept of "the critical time" discussed earlier. Many bacteria are capable of initiating growth at room temperature, the temperature at which plates are usually inoculated. If the plates are preincubated at 35°C, the effect will be exaggerated. Studies examining this phenomenon have been used to elucidate the critical time (3). With two exceptions, disk diffusion methods recommend that the disks be applied within 15 minutes of plate inoculation.

Incubation Time

With rapidly growing bacteria, the zone diameter is formed within a few hours of commencing incubation (3). In theory, therefore, it is possible to read zone diameters when growth becomes visible. With further incubation—recommended for all organisms in all methods—there can be subtle changes in the zone diameter beyond the time when growth first becomes visible. These changes are the result of (a) delayed growth, (b) better visualization of partial inhibition, and/or (c) the delayed appearance of resistant variants. As zone diameter breakpoints are applied to species after specified incubation periods, it is not generally possible to use these values to determine susceptibility earlier. However, if the zone of inhibition is clearly in the resistant range, further incubation is only likely to make the zone smaller, and, therefore, it would be possible to categorize a strain as resistant when growth becomes visible. Incubation of the plate for a few hours longer than typically recommended (e.g., Enterobacteriaceae for 24 rather than 18 hours) will not usually influence the interpretation significantly. Some bacteria need longer intervals of incubation than 24 hours, such as *H. pylori* (3 days) and some strains of *Yersinia pestis* (48 hours).

A specific incubation duration of 24 hours is recommended in many methods for staphylococci when tested against the antistaphylococcal penicillins, usually represented by oxacillin or methicillin, and the glycopeptides, represented by vancomycin. In parallel with broth-based susceptibility testing methods, which can use 2% NaCl, this duration is used in order to maximize the expression of strains with heteroresistance. It must be used in consort with an incubation temperature of 35°C (and no greater). In the BSAC disk method, incubation at 30°C and the addition of 2% NaCl are also used, as these factors are known to enhance heteroresistance expression (90,91). Incubation for 24 hours is also recommended for enterococci when testing them against vancomycin in the CLSI method (27).

Incubation Temperature

Incubation temperatures are designed to optimize the growth of the organisms under test. Most human bacterial pathogens are adapted to optimum growth at 37°C. Most methods therefore recommend growth at 35°C to 37°C. Incubation at 30°C is known to enhance the expression of heteroresistance to methicillin and other antistaphylococcal penicillins (90) and has been recommended as part of the recently developed BSAC disk susceptibility test (12).

Inoculum Density

Inoculum density probably has a greater effect on the ultimate zone diameter than any other variable. As described by Barry (84), the critical population is one of the three critical parameters in determining zone size. Higher inoculum densities will result in bacterial numbers reaching the critical population sooner, at a time when the critical concentration has diffused less than with lower inoculum densities (84). In the case of very dense inocula, it is likely that no zone at all will be formed, even if the organism is susceptible. Inoculum density is particularly important when the bacteria produce inactivating enzymes such as β-lactamases, especially if the enzyme requires induction, as in the case of staphylococcal