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Antimicrobial Susceptibility Testing

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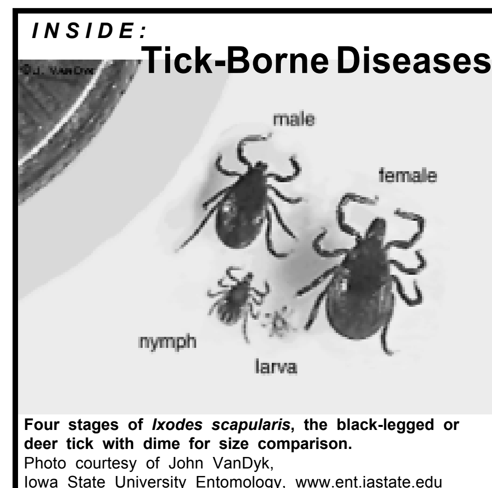
Introduction

The emergence of clinically significant resistance to antimicrobial agents has resulted in the increased importance of antimicrobial susceptibility testing of a wide variety of bacterial species. For example, strains of *Streptococcus pneumoniae* with reduced susceptibility to penicillin, which were not thought to be prevalent in the U.S. until the late 1980s, are now estimated to account for 30-40% of all isolates.[1] Many facilities are faced with high rates of oxacillin (methicillin) resistant *Staphylococcus aureus* (ORSA, MRSA) colonization and infection among their patients. In addition, vancomycin-resistant enterococci, and *Klebsiella* harboring extended-spectrum β-lactamases have become increasingly prevalent in the acute care setting. This article will outline the methodologies currently used in the Microbiology Laboratory at MGH for identifying resistant organisms and will highlight organisms for which susceptibility testing is especially important.

Antimicrobial Susceptibility Testing Methods

Tube Dilution, Microdilution, and Rapid Vitek Test Methods

One of the earliest methods of antimicrobial susceptibility testing was the tube dilution method. This procedure involves preparing two-fold dilutions of antibiotics in a liquid bacterial growth medium. Typically, eight or more concentrations of a drug are prepared in a final volume of 1 to 2 ml per tube. The antibiotic containing tubes are then inoculated with a standardized bacterial suspension. After overnight incubation, the tubes are examined for macroscopically visible evidence of bacterial growth in the form of turbidity. The lowest concentration of antibiotic that prevents visible growth represents the endpoint of the test or the minimal inhibitory concentration (MIC).[3] The modification of the tube-dilution test that has popularized dilution testing is the miniaturization afforded by the use of small, disposable plastic microdilution trays for performance of susceptibility tests. Such trays ordinarily contain 96 wells, each with a volume of 100 µL, which



Four stages of *Ixodes scapularis*, the black-legged or deer tick with dime for size comparison.
Photo courtesy of John VanDyk, Iowa State University Entomology, www.ent.iastate.edu

allows ~12 antibiotics to be tested in a range of eight two-fold dilutions in a single tray.[3] The most recent variation of the tube dilution test utilizes even smaller test modules and highly computerized equipment such as the Vitek System (BioMérieux-Vitek, Hazelwood, MO). The Vitek system was originally designed for use in space exploration efforts of the 1970's as an onboard testing system for U.S. spacecraft. This highly automated, miniaturized system allows for repetitive turbidimetric monitoring of bacterial growth during an abbreviated incubation period. The test cards used at the MGH have 45 wells containing 16 different antimicrobial agents (fewer dilutions of a single antimicrobial agent than in microdilution trays) and are the size of a playing card. The Vitek system allows testing of the common, rapidly-growing gram-positive and gram-negative aerobic bacteria, with results available in a period of 4 to 10 hours. The potential clinical benefit of performing antimicrobial susceptibility tests in 4 to 10 hours rather than after an overnight incubation has been difficult to measure. Several studies have shown cost savings and decreased mortality. These benefits require aggressive laboratory reporting to bring the results to the attention of clinicians for appropriate action.[4] The advantages of the automated susceptibility test instruments include enhanced reproducibility of results and the ability to interface the instrument with the computer, thereby decreasing transcription errors. The main disadvantages are the inflexibility of the drug arrays available in the standard commercially prepared antibiotic panels and the inability to test more fastidious bacteria.[3]

Disk Diffusion Testing - One of the simplest and most reliable susceptibility testing methods is the disk diffusion or Bauer-Kirby procedure. This method has been widely used and well standardized over a number of years. The test is performed by applying a standardized inoculum of ~1-2 x 10^8 CFU/mL to the surface of a large (150-mm diameter) Mueller-Hinton agar plate. Up to 12 commercially prepared fixed concentration filter paper antibiotic disks are placed on the inoculated agar surface. Plates are incubated for 16-18 hours in ambient air at 35°C before the results are determined. The diameters of the zones of growth inhibition around each of the antibiotic disks are then measured. The diameter of the zone of inhibition is related to the susceptibility of the isolate and to the rate of diffusion of the drug through the agar medium. The zone diameter correlates inversely with the approximate MIC for that antibiotic. In practice, the results of the disk diffusion test are interpreted by comparing the measured zone diameter (in mm) with the interpretive criteria in tables published by the National Committee for Clinical Laboratory Standards (NCCLS).[2] The results of the disk diffusion testing are qualitative in that a category of susceptibility (susceptible, intermediate, or resistant) is derived from the test rather than a MIC. A principle advantage of the disk

Table 1. Organisms and the Antibiotic Susceptibilities Evaluated at the MGH

Organism Group	Test Method(s)	Antimicrobial Agents Tested
Enteric gram-negative rods and *Acinetobacter anitratus* -urine specimens	Vitek; disk	Amoxicillin-clavulanate, Ampicillin, Aztreonam, Cefpodoxime, Ceftazidime, Gentamicin, Levofloxacin, Nitrofurantoin, Piperacillin, Tetracycline, Trimethoprim-sulfamethoxazole
Enteric Gram-negative Rods and *Acinetobacter anitratus* - non-urine specimens	Vitek; disk	Amikacin, Ampicillin, Aztreonam, Cefazolin, Cefpodoxime, Ceftazidime, Ceftriaxone, Gentamicin, Levofloxacin, Meropenem, Piperacillin, Trimethoprim-sulfamethoxazole
Pseudomonas aeruginosa and other non-enteric gram negative rods	Disk; MIC	Amikacin, Aztreonam, Ceftazidime, Ciprofloxacin, Gentamicin, Levofloxacin, Meropenem, Piperacillin, Ticarcillin, Tobramycin, Trimethoprim-sulfamethoxazole, Chloramphenicol
Staphylococci	Disk; MIC	Penicillin, Oxacillin, Vancomycin, Erythromycin, Clindamycin, Tetracycline, Levofloxacin, Trimethoprim-sulfamethoxazole, Nitrofurantoin, Gentamicin, Rifampin, Linezolid
Enterococci	Disk; MIC	Ampicillin, Vancomycin, Erythromycin, Tetracycline, Levofloxacin, Nitrofurantoin, Doxycycline, Chloramphenicol, Rifampin, Quinupristin/dalfopristin, Linezolid
Streptococcus pneumoniae and viridans group streptococci	MIC; disk	Penicillin, Vancomycin, Chloramphenicol, Clindamycin, Erythromycin, Tetracycline, Trimethoprim-sulfamethoxazole, Ceftriaxone, Levofloxacin
Haemophilus spp.	Disk	Ampicillin, Cefixime, Ceftriaxone, Cefuroxime, Chloramphenicol, Levofloxacin, Meropenem, Trimethoprim-sulfamethoxazole
Neisseria gonorrhoeae	Disk	Ciprofloxacin, Ceftriaxone, Penicillin, Tetracycline, Spectinomycin

diffusion methodology is that it affords flexibility in the panel of antibiotics that can be tested. The primary limitation of the technique is the limited spectrum of organisms for which this method of testing has been standardized. [3]

Antibiotic Panel Selection - The number of agents that can be routinely tested for each organism is limited by the susceptibility testing method used. Typically, test methods allow susceptibility testing of only 9 to 16 different antibiotics. Thus, careful selection of the antibiotic panel is necessary to maximize the usefulness of the results. The antimicrobial agents routinely tested for each category of organism at the MGH are shown in **Table 1**.

Interpretation of Test Results - In the majority of cases, there is no objective evidence that reporting of the quantitative MIC result is any more clinically relevant than reporting the categorical result of susceptible, intermediate or resistant.[5] Further, the clinical indications for the use of MIC results are not well agreed upon. In the treatment of chronic infections such as infective endocarditis or osteomyelitis, for which therapy is likely to be protracted, the reporting of MIC values may be helpful in selecting among a group of similar drugs. For virtually all other infections, categorical results generally provide the necessary information to select the appropriate therapy.

The categorical result of "susceptible" indicates that an infecting organism should be eradicated by therapy with that antibiotic at the dosage normally recommended for that type of infection and species. Conversely, "resistance" to an antibiotic indicates that growth of the organism should not be inhibited by concentrations of the antibiotic achieved with the dosages normally used. An "intermediate" result indicates that a microorganism falls into a range of susceptibility for which the MIC approaches or exceeds the concentration of the antibiotic that can ordinarily be achieved and for which a clinical response is less likely than with a susceptible strain. Exceptions can occur if an antibiotic is highly concentrated in a body fluid such as urine or if higher than normal dosages of an antibiotic can be administered safely (as is the case with some penicillins or cephalosporins).

Problem Organisms

Oxacillin-resistant Staphylococcus aureus (ORSA) - Staphylococci continue to be among the most common of all nosocomial and community acquired bacterial pathogens. Oxacillin (methicillin) resistant *Staphylococcus aureus* was first identified in Boston in 1968.[6] At present, approximately 20 to 25% of *S. aureus* isolated from hospitalized patients in the U.S. are resistant to oxacillin. Both *S. aureus* and coagulase-negative species of *Staphylococcus* may become oxacillin resistant due to the production of a special, low affinity penicillin binding protein, PBP 2a. Production of PBP 2a results in broad resistance to semi-synthetic penicillins, cephalosporins and carbapenems. Oxacillin resistant strains are often (but not always) multiply resistant to several other drug classes including macrolides, clindamycin, aminoglycosides, chloramphenicol, fluoroquinolones, and trimethoprim/sulfamethoxazole. Although previously isolated primarily from infections among hospitalized patients, they are being increasingly reported as the cause of community-acquired infections, especially in children.

The most definitive method for detection of methicillin resistance is now recognized to be the detection of the gene (*mecA*) that encodes production of PBP 2a.[7] This can be accomplished by a polymerase chain reaction method and the use of a direct DNA probe. Genetic testing for the *mecA* gene is clinically useful in the situation where borderline results are obtained by the usual methods. For example, oxacillin resistant isolates with borderline MICs may either contain the *mecA* gene or produce high levels of a β-lactamase that slowly hydrolyzes oxacillin. While vancomycin would generally be the drug of choice for a *mecA* containing strain, those producing high levels of β-lactamase can be treated effectively with penicillinase stable penicillins such as nafcillin.

Vancomycin-resistant enterococci - The prevalence of vancomycin-resistant enterococci (VRE) has increased sharply in the U.S. in the last decade. Vancomycin had been in clinical use for > 30 years before significant levels of VRE were observed. Acquisition of VRE by hospitalized patients has been associated with increased length of stay, underlying disease, intensity of antibiotic exposure, and exposure to broad-spectrum cephalosporins, metronidazole, vancomycin, and other agents. Vancomycin acts by binding to the peptidyl-D-alanyl-D-alanine termini of peptidoglycan precursors and preventing bacterial cell wall synthesis. Enterococci develop resistance to vancomycin by altering their petidoglycan precursors such that glycopeptide antibiotics can no longer bind. VRE are divided into resistance phenotypes based primarily on the patterns of resistance to specific drugs and usually have MICs in the range of 32 to 512 µg/ml.[8] *VanA* and *vanB* phenotypes occur primarily in *Enterococcus faecalis* and *Enterococcus faecium*. *VanC* resistance is reported for *Enterococcus gallinarum* and *Enterococcus casseliflavus*, which demonstrate intrinsic low-level resistance (MICs 8 to 30 µg/mL) to vancomycin.

Vancomycin Intermediate Staphylococcus aureus (VISA) - First reported in Japan, *S. aureus* strains with reduced susceptibility to vancomycin (vancomycin intermediate *S. aureus* or VISA) now have been documented in the United States. Such strains have MICs to vancomycin ranging from 8 to 16 µg/mL. In each of the reported cases, the patients had received long-term, multiple courses of vancomycin or teicoplanin in the 6 months prior to infection with VISA to treat ORSA-associated infections. Surveillance cultures obtained from patient contacts did not grow VISA. The *vanA* and *vanB* genes that are involved in enterococcal resistance to vancomycin do not appear to be involved in this resistance mechanism.[8] The exact mechanism for the reduced susceptibility has not yet been determined but may involve thickening of the bacterial cell wall. The MGH currently screens for VISA by performing a vancomycin MIC on all non-respiratory isolates of ORSA and on any isolate (oxacillin-resistant or susceptible) with a borderline zone of inhibition around the vancomycin disk. Thus far at the MGH, VISA organisms have not been identified.

Streptococcus pneumoniae - Pneumococcal infections are among the leading causes of illness and death worldwide and affect primarily children, patients with co-morbid conditions, and the elderly. The emergence of strains of *Streptococcus pneumoniae* with high level resistance to penicillin (MICs of ≥2 µg/mL) and other antimicrobials is a serious healthcare concern.[10] A unique characteristic of the emerging resistant strains is their involvement in infections occurring primarily in the community setting. This is in contrast to other organisms such as VRE and MRSA that initially emerged as

nosocomial infections. Penicillin resistance is due to altered penicillin-binding proteins and is not mediated by β-lactamase. The development and spread of strains with reduced susceptibility to penicillin is thought to be in part due to the inappropriate use of antimicrobial agents.

Streptococcus pneumoniae with reduced susceptibly to penicillin can be categorized as having high or low level resistance to penicillin. An oxacillin disk diffusion test is used to screen for penicillin resistance. A zone of inhibition greater than 20 mm indicates that the strain will be susceptible to other relevant β-lactam antibiotics including amoxicillin and most cephalosporins. If the oxacillin zone is less than 20 mm, MIC testing is performed to determine if the isolate is resistant, intermediate or borderline susceptible to penicillin. Although extremely rare, resistance to third generation cephalosporins such as ceftriaxone has also been found. At the MGH, MICs for penicillin and ceftriaxone are performed as soon as possible on all blood and CSF isolates. In addition, the disk diffusion test (or microdilution) is used to assess susceptibility to the macrolides, trimethoprim-sulfamethoxazole, clindamycin, vancomycin, tetracycline, and levofloxacin for all isolates.

Enterobacteriaceae with Extended Spectrum β-Lactamases - Virtually all strains of *Klebsiella pneumoniae* produce a plasmid-mediated β-lactamase known as SHV-1, which normally results in resistance to only ampicillin, ticarcillin and piperacillin. Similarly, a substantial percentage of *Escherichia coli* isolates also produce a plasmid mediated β-lactamase, known as TEM-1, that results in ampicillin resistance. Spontaneous mutations may occur in these common enzymes that extends the spectrum of hydrolysis to include later generation penicillins, cephalosporins, and aztreonam. These enzymes are now referred to as extended-spectrum beta-lactamases (ESBL), and they have been subjected to enzymology and genetic sequencing techniques to characterize the precise mutations and unique hydrolytic activities of each enzyme.[9] The currently described unique extended-spectrum β-lactamases are SHV-2 though SHV-23 and TEM-2 through TEM-69. These enzymes all appear to be capable of hydrolyzing penicillins, cephalosporins, and aztreonam and have been associated with adverse outcomes in patients treated with those agents, and in nosocomial infection outbreaks.

To screen for the presence of ESBLs in *Klebsiella* spp. and *E. coli*, susceptibility testing is performed on the drugs that are most readily hydrolyzed by EBSL. These include ceftazidime, aztreonam, cefotaxime, and ceftriaxone. MICs of 2 to 8 μg/mL in this group of drugs are presumptive evidence of an ESBL, since this level of resistance is very unlikely in *Klebsiella* or *E. coli* strains possessing only native SHV-1 or TEM-1 enzymes. To confirm the presence of an ESBL, an isolate is tested for the presence of a substantially lower MIC (or larger zone of inhibition) in the presence of a cephalosporin plus the β-lactamase inhibitor clavulanic acid vs. the cephalosporin alone. When an ESBL has been identified, the susceptibility report for all penicillins, aztreonam, and true cephalosporins (but not the cephamycins, i.e. cefotetan, cefmetazole, and cefoxitin) is changed to resistant.

The Future of Antimicrobial Susceptibility Testing

Recent advances in molecular biology have resulted in novel and definitive methods for the detection of bacterial resistance. Genetic testing for bacterial resistance genes offers the potential for an accurate, sensitive method to directly detect bacterial resistance. Recent success in the detection of the *mecA* gene (the gene responsible for methicillin resistance in *Staphylococcus* species) and the *vanA* and *vanB* genes (the genes conferring vancomycin resistance in enterococci) demonstrates the promise of this approach. Potential limitations of this approach include the large number of resistance genes that would need to be detected and the possibility that certain genes that may be detected may not result in phenotypic resistance. A recent advance that may overcome some of these limitations is the application of genetic biosensor technology to resistance testing. Utilizing this methodology, a large number of resistance genes could be efficiently screened for simultaneously. In the future, this type of analysis may provide a highly definitive, genetic basis for determining antibiotic susceptibility. However, for the present, phenotypic methods remain the only practical and reliable means to assess antimicrobial susceptibility to a wide variety of agents.

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Three Tick-Borne Diseases in the Northeastern United States: Lyme Disease, Babesiosis, and Ehrlichiosis

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Introduction

The tick-borne diseases increase in prevalence as a result of outdoor activities during the summer months. Diagnosis of the tick-borne diseases—borreliosis, ehrlichiosis and babesiosis—represent challenges to the clinician because the conditions include overlapping symptoms, and accurate diagnosis is paramount for effective management. Three tick borne diseases found in New England and the approach to their diagnosis are reviewed in this article. Routine evaluation of stained peripheral blood smears represents an important part of the diagnosis. Due to difficulties in cultivating these fastidious organisms, serologic studies and PCR-based testing can be highly valuable in establishing a diagnosis.

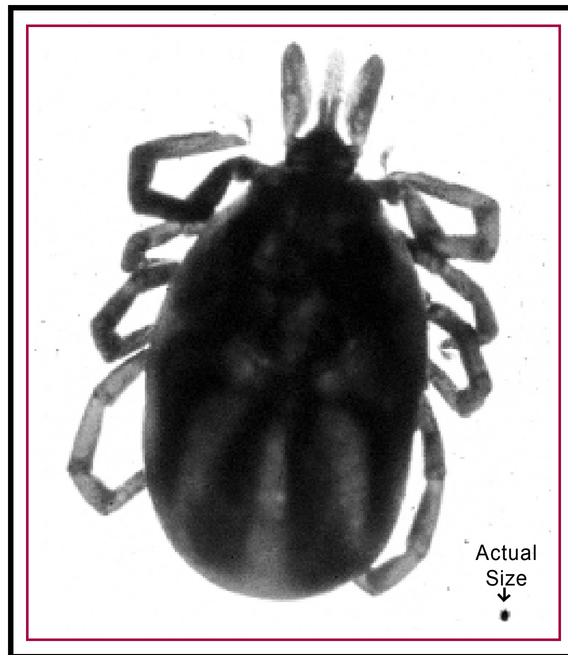
LYME DISEASE

Overview

Causative Agent - Lyme disease (borreliosis) is caused by the motile, flagellated spirochete, *Borrelia burgdorferi*. *B. burgdorferi sensu lato* has been divided into three genospecies: *B. burgdorferi sensu strictu*, *B. garinii*, and *B. afzelii*. *B. burgdorferi sensu strictu* accounts for the majority of cases in the USA. *B. garinii* and *B. afzelii* are usually found in cases from Europe and Asia.

Transmission - *B. burgdorferi* is transmitted by several tick vectors, *Ixodes scapularis* in the eastern and north-central United States, *Ixodes pacificus* in the western United States, *I. ricinus* in Europe and possibly *I. persulcatus* in Japan. Other ticks may also harbor *B. burgdorferi* but encounter humans less frequently.

Ixodes ticks (also known as black-legged ticks, deer ticks, or bear ticks) are "hard" ticks and easily identified by a characteristic "U" shaped groove anterior to the anus. Hard ticks have mouth parts that extend outward, which makes them easily visible when looking down at the specimen. They also have a dorsal hard plate, known as a scutum. *Ixodes* species proceed through three developmental stages: larval, nymphal, and adult. Nymphs in the north feed on small mammals, especially rodents, deer, and birds, but will also attach to larger mammals, including pets and humans. Adult ticks prefer deer but also feed on humans. Ticks of all stages can harbor spirochetes and transmit the disease. The nymphal form of the tick is the most likely to transmit the disease because it is active in the spring and summer, coinciding with outdoor activities. Ticks require a period of attachment of at least 24 hours before they can transmit an infectious dose. There is no known human to human transmission of *B. burgdorferi*, although vertical transmission has been proposed.



Clinical Symptoms - Untreated Lyme disease usually proceeds in three basic stages. In stage 1 (Early-Localized infection), patients develop a characteristic skin rash known as erythema migrans. A macule or papule at the site of the tick bite expands gradually as an erythematous region with partial central clearing. Erythema migrans is commonly found on the axilla, groin, and thigh but may be anywhere. Approximately 25% of infected patients lack erythema migrans. Many patients also experience flu-like symptoms such as fatigue, fever, and myalgias. Regional lymphadenopathy is common. In stage 2 (Early-Disseminated infection), which occurs 1-4 months after infection, patients may experience neurologic manifestations such as radicular pain and fluctuating symptoms of meningitis. The most common cardiac abnormality is a varying degree of atrioventricular block. Musculoskeletal pain is common in this stage and consists of migratory

Deer Tick - Courtesy of Domenic R. Misiano; Photograph by Michelle Forrestall, MGH Pathology Photo Services

pain in the tendons, muscles, bones, and joints. Patients may also develop secondary annular cutaneous lesions similar to the initial skin lesion. Stage 3 (Late-Persistent infection) patients exhibit intermittent attacks of chronic arthritis in large joints, especially the knees. Symptoms can include encephalopathy affecting memory, mood, or sleep. These symptoms may also be accompanied by distal paresthesias or spinal radicular pain.

Laboratory Diagnosis

Direct Detection - Direct detection of organisms in tissue or body fluids lacks sensitivity and is rarely useful in the diagnosis of infected patients. *B. burgdorferi* infection rarely results in microscopically visible spirochetemia. *B. burgdorferi* may be visualized in tissue (e.g., lymph node) stained by the Steiner or Warthin-Starry procedure. These stains are available as special stains in tissue sections in the MGH Histology Laboratory.

Table I: Testing Available for Suspected Lyme Disease

Test	Required Sample
Direct visualization	
Smear analysis by silver stain (e.g., Warthin-Starry, Steiner)	Tissue specimen
Cultivation	Tissue specimen*
Serology	
ELISA or IFA, Immunoblotting	Peripheral blood (red top) or CSF**
PCR	
PCR	Peripheral blood (purple top), CSF, urine, synovial fluid, or tissue specimen

* Not generally available (not available at MGH)
** CSF specimens must be accompanied by a serum sample (red top tube)

Cultivation - Although *B. burgdorferi* may be cultured in the laboratory in specialized media, clinical microbiology laboratories are rarely prepared to cultivate spirochetes. Resuspended plasma, CSF, or a macerated tissue biopsy is placed into modified Kelly's medium, capped and incubated at 33°C for 6 weeks or longer. Subcultures (0.1 mL) are taken weekly from the lower portion of the broth, placed in fresh media, and examined by dark-field microscopy or acridine orange-staining for the presence of spirochetes. Culture of *Borrelia* organisms, although possible, is technically challenging and rarely offered in routine diagnostic settings.

Serologic Tests- Serologic studies represent the most common approach for screening and establishing the diagnosis of Lyme borreliosis in patients. Serodiagnosis is particularly important as culture-based strategies and direct antigen testing are not feasible. However, the specificity of serologic tests is less than desirable due to the presence of conserved epitopes in spirochetes of normal flora. In 1994, the Second National Conference on the Serologic Diagnosis of Lyme Disease recommended a two-step approach.[1]

Screening for total antibodies to *B. burgdorferi* is a relatively rapid and inexpensive approach. Indirect immunofluorescence (IFA) or enzyme immunoassay (EIA), represents an initial diagnostic test selection. If the total antibody screening test is positive or equivocal, the specimen is tested by immunoblot studies (Western blot). Both IgM and IgG can be evaluated by immunoblot. Persons with late stage or disseminated Lyme disease usually have a robust IgG response. If it is early in the illness, a positive IgM result alone is not recommended for use in determining active disease because of the likelihood of false-positive results. If a patient with a suspected early stage Lyme disease has a negative serology, retrospective evidence of infection is best obtained by testing paired acute and convalescent serum samples.

At the MGH, serologic screening is performed by EIA testing for IgG and IgM serum antibodies. If the EIA is positive, specimens are sent to Imugen (Norwood, MA) for immunoblotting. At Imugen, IgG immunoblot studies are performed with two different *B. burgdorferi* laboratory strains (G39/40 and 49736). The normal range is serum reactivity to less than five *Borrelia* antigens on each blot. IgM, IgG, and IgA antibodies to *Borrelia* are also measured at Imugen by capture enzyme immunoassay.

PCR - Although not commonly performed, PCR-based testing is available in several medical center laboratories. Outer surface protein (Osp A or Osp B), flagellin, or 16S rRNA genes represent target sequences for PCR-based diagnosis. Qualitative PCR–based testing may be clinically useful for the diagnosis of neuroborreliosis in patients with an established serologic diagnosis. Real-time PCR techniques may present a method for quantifying *B. burgdorferi* in tissue samples.[2] At the MGH, CSF or tissue specimens may be submitted to the Mayo Medical Laboratories, which can detect *B. burgdorferi* DNA by PCR amplification.

Treatment

Early stage Lyme disease can be treated with doxycycline, amoxicillin, azithromycin, or cefuroxime for 2-3 weeks. Advanced stages of the disease are difficult to treat. Intravenous ceftriaxone or penicillin for 2-3 weeks may be used.

Vaccine

In 1998, LYMErix (SmithKline Beecham Pharmaceuticals) became the first vaccine approved for the prevention of Lyme disease in the United States. It is approved for use in people from ages 15-70 and is given in three doses of 0.5 mL each, at 1 month, and 12 months after the initial dose. The duration of immunity is not yet known.[3] Use of the vaccine in pediatric populations remains controversial and has not been approved by the FDA.

BABESIOSIS

Overview

Causative agent - The causative organism of babesiosis was first described by Babes in 1888 while investigating hemoglobinuria in cattle in Romania. Only a few of the many species of *Babesia* are known to infect humans. In New England and the eastern United States, the disease is caused by *Babesia microti* while in California, it is caused by *B. equi*. In Europe, the disease is caused by *B. divergens* and *B. bovis*. *B. canis* has been found to be responsible for several cases in Mexico and France.

Transmission - *Babesia microti* is transmitted by the tick *Ixodes scapularis* in the northeastern United States. The larvae of the tick feed mainly on the white-footed mouse (*Peromyscus leucopus*). When larvae develop into nymphs and adults, they feed on the white-tailed deer (*Odocoileus virginianus*), but may also choose a human host. In the 1970s many cases were reported from the coastal areas and islands of New England, especially Nantucket. The organism has also been transmitted via blood transfusion from asymptomatic donors.[4]

Clinical Findings - The incubation period is approximately 7-21 days. The clinical presentation is variable, ranging from asymptomatic to rapidly progressive and fatal. Some patients experience a gradual onset of malaise, anorexia and fatigue. Fever with a drenching sweat is also very common. More severe disease occurs in people who have undergone splenectomy, the elderly, and immunocompromised individuals. The disease course is characterized by high fever, massive hemolysis, hemoglobinemia, and hemoglobinuria.

Laboratory Diagnosis

Routine Laboratory Tests - Patients often present with hemolytic anemia. The reticulocyte count may be increased with increased with elevated LDH, increased indirect bilirubin, and decreased haptoglobin levels. Leukopenia is uncommon, although lymphopenia occurs in most patients and atypical lymphocytes are usually present.[5] The erythrocyte sedimentation rate (ESR) is usually elevated, in addition to elevated hepatic transaminases and alkaline phosphatase levels.

Direct Visualization - Two rapid screening methods are used for the identification of *Babesia* organisms. Field's Test is a rapid method performed with a thick peripheral blood film. Erythrocytes in the film are lysed and stained with methylene blue, azure B, and eosin. The method dehemoglobinizes and stains in less than 15 seconds. This rapid screening method is being replaced by the quantitative buffy coat method (QBC) method.

The QBC was initially developed for detection of malaria parasites. However it can also be used as a screening test for babesiosis and is at least as sensitive as peripheral blood smear analysis.[6] This method entails centrifuging about 65 µL of the patient's blood in a thin capillary tube with a coating on its wall of acridine orange (AO) stain. AO stains nucleic acid. Denser, infected erythrocytes concentrate with the rest of the red blood cells and are detected by fluorescence microscopy.

The gold standard for identification of *Babesia* is the visualization of the intraerythrocytic organisms in thick or thin blood films. *Babesia* organisms can be made visible by Giemsa staining of peripheral blood smears. The organisms appear as intraerythrocytic oval ring structures (1-3 µm) with pale blue cytoplasm and one or two tiny, red dots. As the *Babesia* organisms mature, they assume an ameboid or piriform morphology, and there can be multiple organisms inside the same cell.

The *Babesia* ring structures can be easily confused with the ring forms in *Plasmodium* infections. There are several features that distinguish *Babesia* ring structures from those of *Plasmodium*. These differences are summarized in **Table 3**. *Babesia microti* organisms occasionally have four to five rings per erythrocyte and sometimes form a tetrad, called a "Maltese Cross." The "Maltese Cross," although not often found, is diagnostic of babesiosis. The individual rings are smaller than those in *Plasmodium* infections and, unlike older *Plasmodium* forms, there is a lack of hemozoin pigments within the affected erythrocytes.

As these organisms may be difficult to detect in peripheral blood, direct visualization lacks sensitivity, producing false negative results with low-level parasitemia. The degree of parasitemia varies between 1% and 20% in patients with a normal, functioning spleen. However it can be as high as 85% in splenectomized patients. In the

Table 2: Testing Available for Suspected Babesiosis

Test	Required Specimen
Direct visualization	
Field's Test Quantitative Buffy Coat Analysis Thick blood film with Giemsa stain Thin blood film with Giemsa stain	Peripheral blood (purple top preferred)
Serology	Peripheral blood (red top) or CSF
Cultivation followed by direct visualization*	Peripheral blood (purple top) or tissue specimen
PCR	Peripheral blood (purple top) or tissue specimen

*Not available in most laboratories (including MGH)

MGH Parasitology Laboratory, all four of these visualization methods are performed with suspected cases of babesiosis.

Cultivation - Successful culture of *Babesia* requires an animal host, making this approach impractical for the diagnosis of infected patients. *B. microti* may be differentiated from other *Babesia* species by intraperitoneal inoculation of Syrian golden hamsters with blood from infected patients. The hamster's blood is then periodically examined weekly for up to 6 weeks for the presence of infection. The infection does not appear in the blood of the hamster until 2-4 weeks after inoculation.[7]

Table 3. Microscopic Features of Babesia

- Smaller than Plasmodium spp.
- No hemozoin pigment in infected erythrocytes
- Multiple organisms in one erythrocyte is possible
- Sometimes form a "Maltese Cross," which is diagnostic

Serologic Tests - Detection of seroconversion with paired acute and convalescent sera, or the presence of an elevated antibody titer in a single specimen may be useful for diagnosis. The indirect immunofluorescence assay (IFA) is antigen specific for *B. microti*. A titer of >1:64 is indicative of seropositivity, and a titer of >1:256 is considered diagnostic of acute infection. The correlation between the level of the titer and the severity of symptoms is poor. Cross-reactivity with other *Babesia* species and malarial organisms has been described in patients with low titers during the acute phase of the illness.[8] Elevated titers can persist for months after the resolution of symptoms, making it difficult to determine if an elevated titer is due to current or past infection. At MGH, patient sera are sent to the state laboratory which refers samples to the Centers for Disease Control (CDC) in Atlanta, Georgia. Only IgG antibody determinations are performed.

PCR - PCR amplification produces a 238 base-pair amplicon. The test is specific for *B. microti*.[9] However, this test is not applicable for screening.[10] Blood or tissue specimens can be submitted for sendout testing via Chemistry laboratory to Mayo Medical Laboratories for PCR-based testing.

Therapy

Antimicrobial therapy is recommended for asplenic, immunodeficient patients, the elderly, and patients with severe infections. The regimen consists of a combination of clindamycin and oral quinine. An alternative regimen is oral azithromycin and oral atovaquone.[11] Exchange transfusion has been proven effective for patients with high-level parasitemia (>10%), severe disease, or massive hemolysis.

Coinfection with Borrelia

Coinfection with *Babesia sp.* and *Borrelia burgdorferi* has been reported in the literature.[12] This report states that approximately 10% of patients with Lyme disease are coinfected with *Babesia* in southern New England. Coinfected patients appear to have a more severe disease course and prolonged convalescence. In particular, coinfected patients may experience severe episodes of intense fatigue as well as more pronounced headaches, chills and sweats. However, the mechanism by which these infections potentiate one another is not yet understood. If patients are infected with either pathogen or are refractory to antimicrobial therapy, diagnostic testing for both agents should be considered.

EHRLICHIOSIS

Overview

Causative Agent - *Ehrlichia* organisms are members of the Rickettsiaceae family and are gram-negative, intracellular, pleomorphic bacilli. The current method of classification divides the *Ehrlichia* species into three genetically related clusters, as shown in **Table 4**.

The three groups are the *E. canis* genogroup, the *E. phagocytophila* genogroup and the *E. sennetsu* genogroup. The *E. canis* genogroup contains the medically important species, *E. chaffeensis* and *E. canis*. The *E. phagocytophila* genogroup contains the species *E. phagocytophila* and *E. equi*, and the *E. sennetsu* group contains the species *E. sennetsu*.

No clearly defined disease syndrome has been described for *E. canis*. *E. sennetsu* causes a self-limited mononucleosis-like illness that has been observed in Japan and Malaysia. Antibiotic treatment is seldom necessary. *E. chaffeensis* causes human monocytic ehrlichiosis (HME). Human granulocytic or granulocytotropic ehrlichiosis (HGE) is caused by an organism very similar to *E. equi* and *E. phagocytophila*, agents of granulocytic ehrlichiosis in horses in California and ruminants in Europe, respectively.[13] HGE may also be caused by another newly discovered species, *E. ewingii*.[14]

Ehrlichia species undergo three developmental stages. Elementary bodies enter a leukocyte by phagocytosis and multiply rapidly. After 3-5 days, small numbers of tightly packed elementary bodies are visible which are called initial bodies. During the following 7-12 days, the initial bodies develop into morula or mulberry forms.

Transmission - The major vector for *E. chaffeensis* is the Lone Star tick, *Amblyomma americanum*. The principal reservoir for *E. chaffeensis* is the white-tailed deer, which hosts all stages of *A. americanum*.

The primary tick vector for the agent of HGE is *Ixodes scapularis* in the eastern United States and *Ixodes pacificus* in California. Additionally, *Dermocenter variabilis* represents a secondary tick vector in the United States. The major reservoir for infection may be the white-footed mouse in the eastern United States.

Clinical Symptoms - Ehrlichiosis is a general term that encompases human granulocytic (HGE) and monocytic (HME) Erlichiosis. These two infections are caused by two different species of Ehrlichia bacteria. Headache and fever, although nonspecific findings, represent the most common clinical abnormalities in patients with either infection.

HGE has an incubation period of approximately 9 days following a tick bite. Approximately 2-11% of patients have a maculopapular and truncal rash upon infection. HME usually presents with fever within one to two weeks of a tick bite. Symptoms include headaches, myalgias and malaise. A petechial, maculopapular or erythematous rash can be seen in approximately one third of patients with HME

and 2%-11% of HGE patients. The rash is more commonly observed in children.

Table 4. Ehrlichia Classification

Species	Disease
E. canis	Canine ehrlichiosis
E. chaffeensis	Human monocytic ehrlichiosis (HME)
E. equi	Equine ehrlichiosis
E. ewingii	Human granulocytic ehrlichiosis (HGE)
E. phagocytophila	Tick-borne fever
E. phagocytophilia-like agent	Human granulocytic ehrlichiosis (HGE)
E. sennetsu	Sennetsu fever

Laboratory Diagnosis

Routine Laboratory Tests - Laboratory abnormalities often include leukopenia, thrombocytopenia and mildly to moderately elevated serum hepatic transaminase levels. Leukopenia most likely occurs as a result of excessive destruction of leukocytes in the peripheral circulation. Leukopenia, thrombocytopenia, and elevated transaminase levels usually resolve within days of successful therapy and can be used to monitor treatment efficacy.

Direct Detection - For HGE, direct observation of intraleukocytic morulae in Wright-Giemsa stained peripheral blood or buffy coat smears is a rapid, inexpensive and readily available laboratory test. Morula-like structures may be visible in cells in the CSF or tissue. Morulae represent eosinophilic cytoplasmic inclusions in circulating granulocytes in HGE. The finding of morulae in neutrophils is proportional to the severity of the illness. Careful examination should reveal morulae in 20-80% of acutely infected individuals. In HME, intraleukocytic morulae are difficult to detect in peripheral blood smears, depending on the skill of the microscopist. Blood smears usually become negative 24-48 hours after the beginning of doxycycline therapy.

The quantitative buffy coat (QBC) method can also be used for diagnosis of either HGE or HME. QBC allows larger volumes of blood to be screened for infected leukocytes.

Cultivation - Laboratory cultivation of *E. chaffeensis* (HME) as well as an *Ehrlichia phagocytophila* -like agent of HGE, have been achieved in the research laboratory and require established cell lines.[15] Clinical microbiology laboratories do not routinely offer culture for *Ehrlichia*. It is rarely available for diagnosis even in large medical centers, such as the MGH.

Serologic Tests - In HGE, the diagnosis is confirmed by seroconversion or by a single serological titer >1:80 in patients with compatible clinical findings. Seroconversion is defined as a 4-fold rise in the titer of paired acute and convalescent sera. Approximately 5-20% of patients with HGE may satisfy the diagnostic criteria for Lyme disease. Ehrlichiosis can mimic aspects of Lyme disease, although erythema migrans represents a finding specific for Lyme disease. False positive serologic results or true coinfection with *Ehrlichia* or *Borrelia* may account for clinical laboratory similarities. In HME, the diagnosis is confirmed by seroconversion or by a serological titer >1:128 in patients with an appropriate clinical picture. At MGH, serum or CSF samples are sent to the state laboratory, which refers them to the CDC for assessment of IgM and IgG antibodies to Ehrlichia.

PCR - PCR-based detection of the *E. phagocytophilia*-like agent of HGE represents the most sensitive and direct approach to diagnosis. Whole blood in purple top tubes (anticoagulated in EDTA) can be submitted for DNA amplification. Genetic targets include 16S rDNA and *epank1* genes. *E. ewingii* may be detected with primers complementary to species-specific 16S rDNA sequences.

PCR-based detection of *E. chaffeensis* includes amplification of sequences within 16S rDNA. The homologous oligonucleotide primers HE1 and HE3, primers complementary to 16S rDNA sequences, do not amplify genomic targets in *E.canis* or *E. phagocytophila*.[16]

Treatment

Both HGE and HME are treated with doxycycline. Since a persistent

Table 5. Testing Available for Suspected Ehrlichiosis

Test	Required Sample
Direct visualization	
Thick blood smear*	Peripheral blood (purple top)
Quantitative buffy coat analysis*	Peripheral blood (purple top preferred)
Serology	Peripheral blood (red top) or CSF
Cultivation*	Peripheral blood (purple top)
PCR	
Human granulocytic Ehrlichiosis (HGE)	Peripheral blood (purple top)
Human monocytic Ehrlichiosis (HME)*	Peripheral blood (purple top)

*Not available in most labs (including the MGH)

symptomatic infection has rarely been documented in human patients, there are no established guidelines for long-term antimicrobial therapy.

Coinfection with Borrelia

Common tick vectors transmit agents of human erhlichiosis, *Borrelia burgdorferi*, and *Babesia microti*. Approximately 10% of patients with human granulocytic erhlichiosis (HGE) also have serological evidence of recent Lyme disease.[17] However, serologic evidence alone is insufficient to verify a dual infection since elevated titers may persist following cure. Reports in the literature have documented direct isolation of both organisms from a single clinical specimen, demonstrating active coinfection.

Summary

Three tick-borne diseases, all caused by different organisms, were discussed in this article. *Borrelia burgdorferi* (spirochete), and *Ehrlichia spp*. represent bacterial pathogens and *Babesia microti* is a sporozoan. However, all of these organisms may be transmitted by the same tick species, *Ixodes scapularis*. Infections with these tick-borne organisms present diagnostic challenges because laboratory cultivation and antigen detection are generally not possible. Serodiagnosis and molecular diagnostics represent important strategies that are becoming more widely available for these pathogens. Coinfection has been reported in the literature and should be considered when a patient is diagnosed with one of these tick borne agents. The expanding menu of available laboratory tests for the detection of these tick-borne organisms will facilitate diagnosis and patient management.

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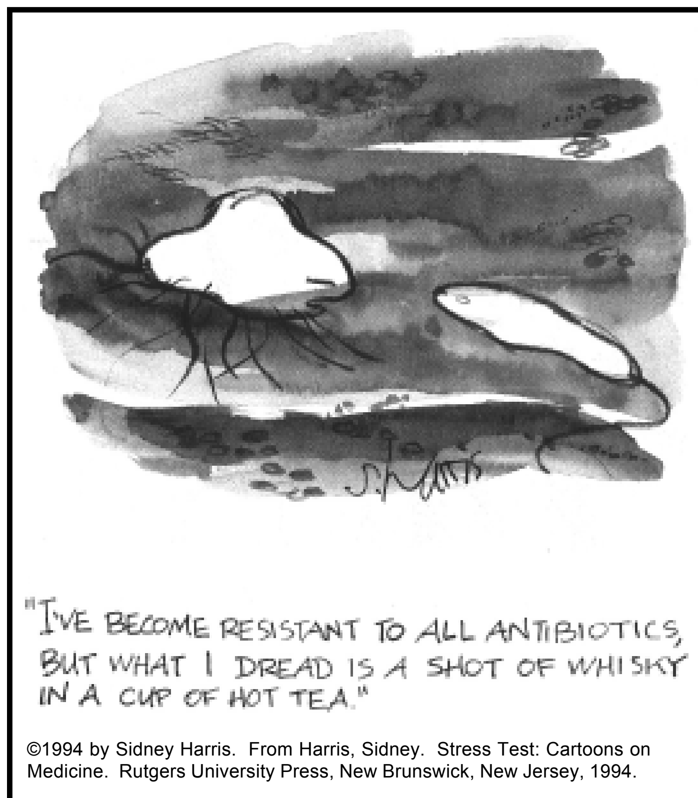

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Clinical Laboratories Update

2001 MGH Laboratory Handbook

If you haven't yet received the 2001 MGH Laboratory Handbook, you can request a copy by calling the 4-LABS Customer Service Center, or by downloading it from our website (see below for details).

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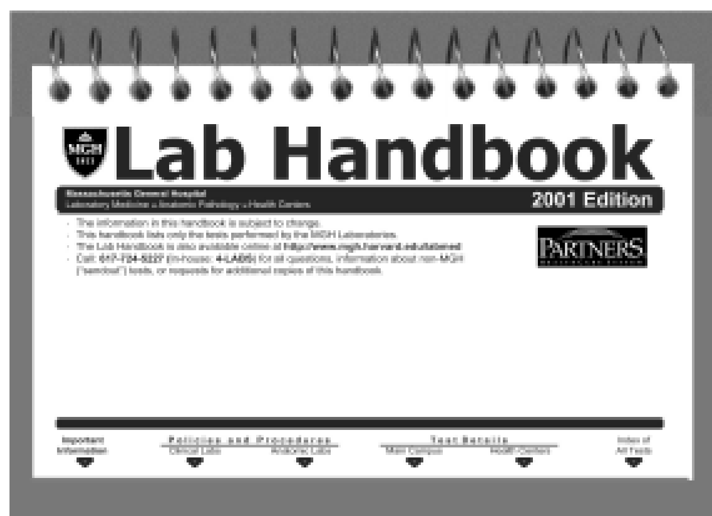

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- We hope to have a searchable version of the online lab handbook by 2002.
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