REVIEWS OF INFECTIOUS DISEASES • VOL. 11, SUPPLEMENT 6 • SEPTEMBER-OCTOBER 1989
 0162-0886/89/1105-0028$02.00

Isolation Techniques for Spirochetes and Their Sensitivity to Antibiotics in Vitro and in Vivo

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Leptospira interrogans can be cultured from blood and cerebrospinal fluid during the first week of leptospirosis and from urine thereafter. Studies of in vitro sensitivity indicate that these organisms are sensitive to most antibiotics. Tetracycline and penicillin G are most often used clinically, although laboratory studies suggest that the bactericidal activity of penicillin G may be inadequate. *Treponema pallidum* cannot be satisfactorily cultured. It is identified by dark-field microscopy. Studies of in vivo sensitivity show that penicillin G is highly active against the syphilis pathogen. Since syphilis and gonorrhea may occur simultaneously, ceftriaxone, which is as active as penicillin G against *T. pallidum* but is also active against penicillinase-producing gonococci, is a logical choice for therapy. *Borrelia burgdorferi* has been cultured from the blood, cerebrospinal fluid, and skin of patients with Lyme disease. In vitro studies have shown tetracycline and erythromycin to be effective against *B. burgdorferi* and penicillin G to be less so, although all are commonly used clinically. Ceftriaxone has also proven to be highly effective in laboratory studies and for clinical treatment.

Spirochetes are flexible, helical bacteria that are grouped together because of common structural features and a unique type of motility. All are also very slow-growing, a factor that may play a role in determining the appropriate duration of antibiotic therapy.

The heterogeneity that exists among the spirochetes becomes apparent when other characteristics such as physiology, habitat, and pathogenicity are considered. For example, there is considerable variability in terms of oxygen requirements, spirochetes covering the full range – from obligate anaerobes to aerobes – although most of the organisms included in this discussion are classified as microaerophilic. The spirochetes of medical importance belong to the genera *Leptospira*, *Borrelia*, and *Treponema*.

Leptospirosis

Leptospirosis is an acute febrile illness caused by serovars of *Leptospira interrogans*. The infection is worldwide in distribution, and its prevalence is related primarily to geographic location and occupation. Leptospirosis is acquired through direct or indirect contact with the urine of leptospiruric animals. Maternal-fetal transmission of the spirochete may also occur.

Leptospirosis is a biphasic illness. The spirochetemic, or acute, phase lasts ~1 week and is followed by the spirocheturic, or immune, phase, which may last weeks to months. The severity of the disease ranges from subclinical to fatal, and the clinical manifestations are many and varied. Accordingly, leptospirosis is often misdiagnosed as aseptic meningitis, hepatic disease, fever of unknown origin, or influenza. Since the clinical manifestations are not pathognomonic, laboratory tests play an important role in the diagnosis of this illness. A definitive diagnosis of leptospirosis requires one of the following: (*1*) isolation of *L. interrogans* from clinical specimens, (*2*) evidence of serologic conversion, or (*3*) a fourfold or greater increase in the titer of agglutinating antibodies.

During the first week of illness (acute phase), leptospires can be isolated from the blood and CSF – blood being the most likely source. Recovery of pathogens from the blood during this phase of the illness is optimized by obtaining samples for culture daily, preferably before the institution of antimicrobial therapy. One to two drops of blood are inoculated into 5 mL of media. Larger inocula are not used because of the presence of factors in the blood that inhibit growth. CSF, which may also contain leptospires during the first week of illness, is processed in the same way as blood.

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A bovine serum albumin–Tween 80 semisolid (0.2% agar) medium [1] is used for the isolation of leptospires. The selective agent fluorouracil (100–200 μg/mL) is added to the medium when potentially contaminated specimens are cultured [2]. Alternatively, contaminated specimens can be injected intraperitoneally into young hamsters or guinea pigs and the leptospires isolated from the blood of moribund animals or from the kidneys 14 days after infection. Isolation media are incubated at 30°C and examined weekly for leptospires. Growth is usually detectable after 2 weeks of incubation, but 6 weeks or longer may be required.

Approximately 1 week after the onset of illness, leptospires can be detected in urine and may continue to be shed for 1 month or longer. Since the leptospires have a relatively short survival time in acid urine, specimens should be cultured as soon as possible—generally within 1 hour. Clean-voided, midstream urine is diluted 1:10 and 1:100 in semisolid media containing fluorouracil. Because the shedding of leptospires is frequently intermittent, several attempts at isolation should be made on different days.

Controversy persists concerning the treatment of leptospirosis. The results of in vitro antimicrobial susceptibility testing of leptospires have varied considerably, and the results of treatment trials for experimental infections have often not been substantiated for naturally acquired infections [3].

Studies of in vitro susceptibility suggest that the various serovars of leptospires are susceptible to all of the commonly used antibiotics except chloramphenicol. However, penicillin G, which has a low MIC, appears to have inadequate leptospiricidal activity. Cook and Thompson [4] not only observed this poor leptospiricidal activity in vitro but reported that penicillin G, although protective for experimentally infected hamsters, lacked adequate curative activity. Although cephalothin, one of the first-generation cephalosporins, was found to have a high level of in vitro activity against leptospires (MIC, 0.03–0.9 μg/mL), a total dose of 1,000 mg/kg was required to prevent death in experimentally infected hamsters [5].

In the most recent study of in vitro susceptibility of leptospires to antimicrobial agents, the MICs of ampicillin, cefmetazole, moxalactam, ceftizoxime, and cefotaxime were lower than the MIC of penicillin G. The MBCs of the third-generation cephalosporins ceftizoxime and cefotaxime were superior to those of penicillin G, streptomycin, tetracycline, ampicillin, and cefmetazole [6]. Alexander and Rule [7] demonstrated the potential usefulness of the newer penicillins and cephalosporins and of ampicillin, chlortetracycline, and doxycycline for the treatment of acute experimental leptospirosis in hamsters. Ampicillin, bacampicillin, mezlocillin, cefotaxime, and moxalactam all possessed both protective and curative activity for the experimentally infected animals. Again, penicillin G was not effective, a finding which points out that although this antibiotic may be highly effective against some spirochetes, i.e., the treponemes, one should not assume that other spirochetes will exhibit similar sensitivity.

Antibiotics most commonly used today for the treatment of human leptospirosis are the penicillins and tetracyclines. However, since leptospirosis has a highly variable clinical course, the effectiveness of antimicrobial therapy has been difficult to evaluate [3]. It is generally believed, however, that the initiation of treatment during the first 4 days of illness will shorten the duration and decrease the severity of the disease. Recently, controlled studies have indicated that doxycycline has both prophylactic and therapeutic value in this regard [8, 9]. Controversy exists concerning the effectiveness of antimicrobial therapy later in the course of the infection. While antibiotics given later in the disease have generally been considered of little clinical value, beneficial results with penicillin G in late leptospirosis have recently been reported [9a].

Syphilis

Syphilis is a complex systemic illness caused by the spirochete *Treponema pallidum*. Because of its protean manifestations, this disease is often referred to as the "great imitator." Transmission usually occurs by sexual contact. A diagnosis of syphilis is based on observations with dark-field microscopy and on epidemiologic, serologic, and clinical findings.

Treponema pallidum cannot be satisfactorily cultured in vitro. Limited multiplication does occur in cell cultures [10], but successful subculturing of this treponeme has not been reported. The pathogen, which is present in the lesions of primary and secondary syphilis, can, however, be isolated from clinical specimens and propagated by the intratesticular injection of rabbits that are free of *Treponema paraluis-cuniculi* infection (i.e., rabbit syphilis). The rabbits should be housed at 16–20°C because the in-

fection develops poorly or is aborted at higher temperatures. The testicular fluid is subsequently examined by dark-field microscopy for the presence of spirochetes.

Since *T. pallidum* cannot be successfully cultivated in vitro, the in vitro sensitivity of these treponemes to antibiotics has most often been determined by assays of inhibition of motility. However, because actively growing spirochetes are not utilized in this test system, it is not truly suitable for the evaluation of most antibiotics.

The cultivable nonpathogenic treponemes have been suggested as models for *T. pallidum* for the purpose of determining antibiotic sensitivities in vitro [11]. However, since genetic studies have established that the nonpathogenic, cultivable treponemes are unrelated to the pathogenic treponemes [12], antibiotic studies with the former would not be expected to be applicable to the latter. When reviewing the earlier literature on treponemal research, it is important to be aware that several of the nonpathogenic, cultivable *Treponema* were often referred to as *T. pallidum*.

A number of workers have investigated the antibiotic sensitivity of *T. pallidum* in vivo using the rabbit as the experimental model [13–16]. The results of these studies have shown that *T. pallidum* is exceptionally sensitive to the action of penicillin G. Tetracycline and erythromycin are alternative agents for the treatment of syphilis. However, the results of treatment of experimental syphilis in rabbits indicate that the therapeutic activity of penicillin G is 50 and 100 times more effective than tetracycline and erythromycin, respectively [13]. Although the evaluation of the therapeutic effectiveness of antibiotics against experimentally induced syphilis in rabbits is lengthy and labor intensive, it is the best assay technique presently available.

We compared the activities of ceftriaxone and penicillin G in rabbits with experimentally induced syphilis [14]. Ceftriaxone was studied because it has a high level of antimicrobial activity, readily penetrates into the CSF and interstitial fluid, and is slowly cleared from the body (half-life of ~8 hours). The activity of these two antibiotics against *T. pallidum* was related to the time required for (*1*) the cutaneous lesions to become dark-field-negative, (*2*) a serologic response to occur, and (*3*) *T. pallidum* to disappear from popliteal lymph nodes. The results verify the exceptional susceptibility of *T. pallidum* to penicillin G. Arnold et al. [15], for example, had reported 50% curative doses (CD_{50}) of penicillin G of 0.6–1.2 mg/kg, or 1,000 to 2,000 U/kg. With the experimental procedure used in our study, a CD_{50} of 0.29 mg/kg, or 480 U/kg, was obtained for this drug. Ceftriaxone was also highly effective against the experimentally induced syphilis, the CD_{50} being 0.96 mg/kg.

Since dual infections with *Neisseria gonorrhoeae* and *T. pallidum* occur and since many *N. gonorrhoeae* strains are penicillin-resistant, it would be advantageous to be able to treat gonorrhea with a drug active against both penicillinase-producing *N. gonorrhoeae* and the syphilis spirochete. Spectinomycin, presently an alternative to penicillin for treating gonorrhea, has low anti-*T. pallidum* activity [16]. Ceftriaxone is highly effective against both penicillinase-producing and non-penicillinase-producing strains of *N. gonorrhoeae* as well as against *T. pallidum*. Thus, ceftriaxone administered as a single dose is effective for the treatment of simultaneous gonorrhea and syphilis.

Infections with *Borrelia*

Borrelia are arthropod-borne microorganisms that infect humans, wild and domestic animals, and birds [17]. The relapsing fevers and Lyme disease are the systemic borrelioses affecting humans.

The etiologic agent of louse-borne relapsing fever is *Borrelia recurrentis*. This disease has primarily been reported in African countries, particularly Ethiopia and the Sudan, and is endemic in the South American Andes.

A number of *Borrelia* species are responsible for tick-borne relapsing fever, which is widely distributed in geographic areas with tropical and temperate climates [18]. Most cases of tick-borne relapsing fever in the United States occur in the western portion of the country and are caused by *Borrelia hermsii*.

Lyme borreliosis, which includes Lyme disease and its related disorders, is presently the most common tick-borne disease in the United States and, perhaps, in the world. *Borrelia burgdorferi* is the etiologic agent of this complex systemic illness with its protean manifestations [19–22].

One of the major breakthroughs in the study of *Borrelia* was the development by Kelly [23] of a culture medium that would support the growth of these organisms. Subsequent modifications of this medium by Stoenner [24] and Barbour [25] resulted in the BSK (Barbour-Stoenner-Kelly) medium, which has

made the isolation of borreliae from a variety of tissues feasible.

Borrelia can now be isolated from their tick vectors by inoculating BSK medium [25] with tissues removed by dissection. A number of tissues of the *Ornithodoros* tick, including the hemolymph, can be cultured for the isolation of the relapsing fever borreliae, whereas *B. burgdorferi* is located primarily in the midgut of *Ixodes* ticks. Alternatively, the tissue suspension can be inoculated into susceptible animals, generally young mice or hamsters, and then isolated from the blood during the first 7 days or from the kidney, spleen, or bladder after 14 days.

One to two drops of blood is added to 10 mL of media. A sample of tissue (10% wt/vol) is then homogenized with a Stomacher Lab-Blender (Tekmar, Cincinnati). After the larger tissue debris is allowed to settle, duplicate 1:10 dilutions of the supernatant are made in the isolation medium. Isolation medium is prepared by the addition of 0.15% agarose (SeaKem LE; FMC Corporation, Marine Colloids Division, Rockland, Maine) to liquid BSK medium. Cultures are examined for spirochetes by dark-field microscopy after 3 weeks of incubation at 30–35°C.

The louse-borne relapsing fever borreliae are present in blood in relatively large numbers during the initial febrile phase of the illness. *B. recurrentis*, however, cannot be satisfactorily cultured in vitro. Isolation of tick-borne relapsing fever borreliae from patients' blood has been infrequent because the diagnosis of relapsing fever is seldom considered initially in the course of disease. When the number of spirochetes is low in patients with relapsing fever, i.e., the organisms cannot be detected visually, the blood can be inoculated into susceptible animals and isolated from these animals as previously described.

Borrelia burgdorferi has been isolated and successfully subcultured from the blood, CSF, and skin of patients with Lyme disease, but this has been a low-yield procedure. The number of *B. burgdorferi* present in the blood early in the infection is low, and they are not visually detectable. The percentage recovery of the organism from blood in two studies ranged from 2% to 6% [20, 26]. Skin biopsy specimens appear to be a better source of the spirochete [26, 27]. These are generally obtained at the leading edge of the erythema chronicum migrans lesions. CSF may be cultured when neurologic manifestations are present. Spirochetes have been isolated from synovial fluid but have not been successfully subcultured [26, 28]. *B. burgdorferi* has not been isolated from the urine of patients with Lyme disease.

At this time there is not a standard method that can be recommended for processing samples for *B. burgdorferi* growth. Although spirochetes can often be detected in culture media after 3 weeks of culture, some isolates may not be visible for several months.

In vitro susceptibility of borreliae to antimicrobial agents. With the development of a medium suitable for the cultivation of *Borrelia*, the antibiotic susceptibility of these spirochetes in vitro could be determined. Barbour et al. [29] studied the action of penicillin G on *B. hermsii*. Medium containing twofold dilutions of penicillin G was inoculated with exponentially growing cells to a final cell concentration of 10^5/mL. The MIC was the lowest concentration of penicillin G at which no growth occurred after 48 hours of incubation at 35°C. Bactericidal concentrations of penicillin G were determined after 48 hours of incubation by inoculating mice with 0.1 mL of the treated cultures and examining the mice for the development of spirochetemia. The MBC was the lowest concentration that prevented spirochetemia. The MIC and MBC for this strain were 0.15 and 1.1 μg/mL, respectively.

Several studies on the in vitro antibiotic susceptibility of *B. burgdorferi* have been reported. Johnson and co-workers evaluated the susceptibility of five strains of *B. burgdorferi* to seven antibiotics [30]. The spirochetes were most susceptible to erythromycin, followed by amoxicillin, ampicillin, doxycycline, and tetracycline. *B. burgdorferi* was moderately susceptible to penicillin G and resistant to rifampin. Berger et al. [27] reported that penicillin, erythromycin, and minocycline were bactericidal to the six skin isolates that were studied. Preac-Mursic et al. [31] determined the antimicrobial susceptibility of seven European isolates of *B. burgdorferi* to 10 agents. The most effective agent was erythromycin, followed by cefotaxime, ampicillin, mezlocillin, and tetracycline. Penicillin G and chloramphenicol were less effective. The spirochetes were resistant to the aminoglycosides gentamicin and amikacin and to trimethoprim-sulfamethoxazole.

Although in the above studies different protocols were used for determining the in vitro susceptibility of *B. burgdorferi*, there was general agreement that erythromycin was the most active in vitro and that tetracycline also possessed good antispirochetal activity. However, Johnson et al. [30] and Preac-Mursic [31] have found *B. burgdorferi* to be only moderately susceptible to penicillin G. All strains of *B. burgdorferi*, whether of human or of tick origin or from Eu-

rope or the United States, displayed a similar level of antimicrobial sensitivity. The three antimicrobial agents commonly used to treat Lyme disease in the clinical setting are tetracycline, penicillin, and erythromycin.

We also investigated the in vitro susceptibility of a human spinal fluid isolate of *B. burgdorferi* to several antimicrobial agents [32]. The broth dilution method was used to determine the MBC. Triplicate tubes containing BSK medium with and without the appropriately diluted antimicrobial agent were inoculated to a final cell density of 10^5 cells/mL. After an incubation period of 3 weeks at 30°C, BSK medium without antimicrobial agent was inoculated with a 10% (vol/vol) inoculum from each of the assay tubes and incubated for an additional 3 weeks. The MBC was the lowest concentration of antimicrobial agent in which spirochetes could not be detected when examined by dark-field microscopy. We decided on the 3-week incubation period for *B. burgdorferi* because the MBC values often increased during weeks 1 and 2 but remained constant thereafter. Considering that *B. burgdorferi* isolates have a generation time of 12–20 hours, the prolonged assay time is reasonable. The use of the relatively large 10% (vol/vol) inoculum produced an antibiotic dilution of 1:60, a concentration that provided a better chance of detecting survivors. The MBCs of the test antimicrobial agents at the end of the 6-week observation period were 0.05 μg/mL for ceftriaxone, 0.13 μg/mL for erythromycin, 1.8 μg/mL for tetracycline, 1.9 μg/mL for amoxicillin, 1.0 μg/mL for doxycycline, 4.0 μg/mL for ciprofloxacin, and 8.7 μg/mL for penicillin G (table 1). *B. burgdorferi* would therefore be considered sensitive to ceftriaxone, erythromycin, tetracycline, amoxicillin, and doxycycline; moderately sensitive to penicillin G; and resistant to ciprofloxacin.

Table 1. MBCs of seven antimicrobial agents for *Borrelia burgdorferi*.

	MBC (μg/mL)	
Antimicrobial agent	Mean	Range
Penicillin G	8.7	3.2–12.8
Amoxicillin	1.9	0.4–3.2
Ceftriaxone	0.05	0.02–0.08
Erythromycin	0.13	0.04–0.16
Tetracycline	1.8	0.8–3.2
Doxycycline	1.0	0.4–3.2
Ciprofloxacin	4.0	0

In vivo susceptibility of borreliae to antimicrobial agents. We also studied the in vivo sensitivity of *B. burgdorferi* [32]. The antibiotics chosen for the study of the in vivo susceptibility of these pathogens were penicillin G, tetracycline, erythromycin, and ceftriaxone. The first three were selected because they are commonly used for the treatment of Lyme disease. Ceftriaxone was included because of its high activity against the pathogen in vitro and its effectiveness in treating experimentally induced syphilis in rabbits [14]. In this study, Syrian hamsters were infected intraperitoneally with *B. burgdorferi* isolated from human spinal fluid [20]. Fourteen days later a regimen of equal daily subcutaneous injections of the test agent for five days was initiated. Fourteen days after the final dose, the hamsters were killed and the kidneys and spleen cultured.

Cultures were examined for spirochetes by dark-field microscopy after 3 weeks of incubation at 30°C. The CD_{50} was calculated by the method of Reed and Muench [33]. The antimicrobial agents with the greatest anti-*B. burgdorferi* activity in vivo were ceftriaxone (CD_{50}, 24 mg/kg) and tetracycline (CD_{50}, 28.7 mg/kg). Erythromycin, which possessed high activity against *B. burgdorferi* in vitro, was ineffective in vivo (CD_{50}, 235 mg/kg). In fact, the highest concentration of erythromycin tested, 403.7 mg/kg, cured only 60% of the test animals. Penicillin G, which had only moderate activity against *B. burgdorferi* in vitro, also displayed poor therapeutic activity in the hamsters; 90% of the hamsters treated with the highest concentration of penicillin G tested, 192.5 mg/kg, remained infected.

Clinical correlation with laboratory studies. Steere et al. [34] reported that tetracycline was the most effective antimicrobial agent for treating early Lyme disease, followed by penicillin and erythromycin. Recently, ceftriaxone was reported to be effective for the treatment of this illness [35]. These results not only correlate with the results of our studies of antibiotic susceptibility in vivo but also demonstrate that the hamster is a useful model for evaluating the activity of antimicrobial agents against *B. burgdorferi*.

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