# *In vitro* susceptibilities of *Leptospira* spp. and *Borrelia burgdorferi* isolates to amoxicillin, tilmicosin, and enrofloxacin

**Doo Kim[1,2], Dorsey Kordick[3], Thomas Divers[1], Yung-Fu Chang[1,*]**

[1]*College of Veterinary Medicine, Cornell University, Ithaca, NY 14853, USA*
[2]*Department of Veterinary Medicine, Kangwon National University, Chunchon 200-701, Korea*
[3]*IDEXX Laboratories, Greensboro, NC 27410, USA*

Antimicrobial susceptibility testing was conducted with 6 different spirochetal strains (4 strains of *Leptospira* spp. and 2 strains of *Borrelia burgdorferi*) against 3 antimicrobial agents, commonly used in equine and bovine practice. The ranges of MIC and MBC of amoxicillin against *Leptospira* spp. were 0.05-6.25 µg/ml and 6.25-25.0 µg/ml, respectively. And the ranges of minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of amoxicillin against *B. burgdorferi* were 0.05-0.39 µg/ml and 0.20-0.78 µg/ml, respectively. The ranges of MIC and MBC of enrofloxacin against *Leptospira* spp. were 0.05-0.39 µg/ml and 0.05-0.39 µg/ml, respectively. Two strains of *B. burgdorferi* were resistant to enrofloxacin at the highest concentration tested for MBC (≥100 µg/ml). Therefore, the potential role of tilmicosin in the treatment of leptospirosis and borreliosis should be further evaluated in animal models to understand whether the *in vivo* studies will confirm *in vitro* results. All spirochetal isolates were inhibited (MIC) and were killed (MBC) by tilmicosin at concentrations below the limit of testing (≤0.01 µg/ml).

**Key words:** amoxicillin, antibiotics, *Borrelia burgdorferi*, enrofloxacin, *Leptospira spp.*, susceptibility, tilmicosin

## Introduction

Leptospirosis is a zoonotic infection with a worldwide distribution that is associated with both endemic disease and epidemics, with the incidence of disease being highest in tropical climates [17]. The number of cases of leptospirosis diagnosed has increased since 1983 in North America [36]. Lyme disease is a multisystemic disorder caused by infection with *Borrelia burgdorferi* and is the most common tick-associated illness in North America [4,7,8].

Leptospirosis and Lyme disease have some overlapping geographic distributions and both can produce acute febrile disease. Although they may be self-limiting diseases in some cases, they can also have significant morbidity and mortality if not prevented or treated. The lack of rapid and accurate diagnostic testing of leptospirosis and Lyme disease often dictate empirical therapy for acute febrile illness. As the differential diagnosis of an acute febrile illness in any particular setting may be extensive, therapeutics that may cover a broad range of infectious diseases are desirable. Doxycycline and penicillin are currently the drugs of choice in the treatment of human leptospirosis, based chiefly on the fact that they are the only agents that have been studied in randomized controlled clinical trials [12,21,29]. Previous studies have shown that the newer cephalosporins and other β-lactams, as well as fluoroquinolone antibiotics, all have good *in vitro* activity against strains of *Leptospira* [26,34]. Doxycyline and tetracycline have also been used in the treatment of equine and canine borreliosis [10,35].

Although many *in vitro* and *in vivo* studies have been performed to find an optimal treatment for spirochetal disease, preferred forms of treatment are still not quite clear. Moreover, the majority of studies concerning *in vitro* susceptibility testing of leptospires and borreliae against antimicrobial agents were conducted with organisms recovered from human infections. The antimicrobial susceptibility of leptospires and borreliae isolated from animals is not well known. In this investigation, *in vitro* antimicrobial susceptibility tests were conducted with 6 spirochetal strains (4 leptospires and 2 borreliae) isolated from animal clinical cases against 3 antimicrobial agents (amoxicillin, tilmicosin, and enrofloxacin), which are used in the treatment of animal diseases.

## Materials and Methods

### Bacterial strains

The four strains of *Leptospira* spp. (*L. kirchneri* serovar Grippotyphosa, *L. interogans* serovar Pomona, *L. interogans* serovar Canicola, and *L. interogans* serovar

─────────────
*Corresponding author
Tel: +1-607-253-3675; Fax: +1-607- 253-3943
E-mail: yc42@cornell.edu

Icterohaemorrhagiae) were obtained from National Veterinary Service Laboratory (USA) and two strains of *Borrelia burdgorferi* (*B. burdgorferei* #15770 isolated from a dog and *B. burdgorferi* #2769 isolated from a horse in our laboratory) were used for this study. All leptospiral and borrelial organisms were not more than 5 passages. Leptospires had been passed through hamsters three times before used. Leptospires were maintained in Ellinghausen McCullough Johnson Harris (EMJH) medium and borreliae were maintained in Barbour-Stoenner-Kelly (BSK)-II medium, as previously reported [5,13].

## Antimicrobial agents

Stock antimicrobial solutions of 1 mg/ml of amoxicillin and tilmicosin, and 0.2 mg/ml of enrofloxacin were prepared with EMJH or BSK-II medium as suggested in National Committee for Clinical Laboratory Standards (NCCLS) document M31-A2 or by their manufacturers [25]. Amoxicillin was purchased from Sigma-Aldrich (USA), enrofloxacin was obtained from its manufacturer (Bayer, USA) and tilmicosin obtained from IDEXX Laboratories (Greensboro, USA). All stock antimicrobial solutions were stored in one-time-use aliquots at −70°C.

## Quality control and internal validity

*L. interrogans* serovar Icterohaemorrhagiae was evaluated by the broth microdilution and macrodilution methods. These methods run 5 times over numerous days with several different EMJH medium lots to determine the reproducibility of our methods. Each replicate run used an individually prepared medium and inoculum suspension. Quality control and internal validity test for *B. burgdorferi* was also conducted with *B. burgdorferi* #2769 strain and BSK-II medium.

## Susceptibility testing for leptospirae

Broth microdilution and macrodilution susceptibility tests were performed with a previously described technique [24]. Testing of each combination of strain and drug was performed in parallel runs by each method, microdilution and macrodilution, to compare the variability of results within and between the methods. Two parallel runs were performed at different times to determine the reproducibility of results. For each

isolate and substance, independent experiments were performed on different days, with minimal inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs) reported as the median of all three experiments.

## Susceptibility testing for Borreliae

Broth macrodilution MICs and MBCs were obtained with a previously described technique [31].

## Statistics

To investigate possible differences in the susceptibility patterns of the spirochetal species tested, the Kruskal-Wallis test was performed for all MICs and MBCs of the antibiotics as determined by our experiments with different spirochetal isolates. The level of significance was $p < 0.05$.

# Results

## Quality control

Quality control (internal-validity) assessment of *L. interrogans* serovar Icterohaemorrhagiae showed consistent results (data not shown). MICs determined by microdilution fell within 2 dilutions of each other for all (100%) of 15 triplicate runs. Fourteen (93.3%) of 15 MICs determined by the macrodilution technique were within 2 dilutions of each other. A single outlier for macrodilution was produced by enrofloxacin. In macrodilution MBCs, all 15 runs (100%) fell within 2 dilutions of each other, although the majority of results for amoxicillin fell outside the upper limit of the drug concentration tested. Quality control assessment of *B. burgdorferi* showed consistent results (data not shown). MICs and MBCs determined by macrodilution fell within 2 dilutions of each other for all (100%) of 15 runs. An assessment of the reproducibility of our technique with a single serovar over numerous days with various lots of media appears to have produced consistent results with the techniques.

## MIC and MBC

MICs and MBCs of each antimicrobial agent for the same isolate spanned a maximum range of +/-1 dilution around the median except *L. interogans* serovar Pomona (Table 1). While the MICs of *Leptospira* spp. determined by the

**Table 1.** *In vitro* susceptibility of 4 strains of *Leptospira* spp. to 3 antimicrobial agents (µg/ml)

| | Amoxicillin | | | Tilmicosin | | | Enrofloxacin | | |
|---|---|---|---|---|---|---|---|---|---|
| | MIC | | Macro MBC | MIC | | Macro MBC | MIC | | Macro MBC |
| | Micro | Macro | | Micro | Macro | | Micro | Macro | |
| *L.* Canicola | 1.56 | 1.56 | 12.5 | ≤0.01 | ≤0.01 | ≤0.01 | 0.10 | 0.10 | 0.10 |
| *L.* Grippotyphosa | 3.13 | 1.56 | 12.5 | ≤0.01 | ≤0.01 | ≤0.01 | 0.20 | 0.10 | 0.10 |
| *L.* Icterohemorrhagiae | 0.78. | 0.78 | 25.0 | ≤0.01 | ≤0.01 | 0.01 | 0.20 | 0.10 | 0.20 |
| *L.* Pomona | 0.78 | 0.78 | 6.25 | ≤0.01 | ≤0.01 | ≤0.01 | 0.20 | 0.20 | 0.39 |
| Range | 0.05-6.25 | 0.10-3.13 | 6.25-25.0 | ≤0.01-0.02 | ≤0.01 | ≤0.01-0.05 | 0.10-0.39 | 0.05-0.20 | 0.05-0.39 |

**Table 2.** *In vitro* susceptibility of 2 strains of *Borrelia burgdorferi* to 3 antimicrobial agents (µg/ml)

|  | Amoxicillin | | Tilmicosin | | Enrofloxacin | |
|---|---|---|---|---|---|---|
|  | MIC | MBC | MIC | MBC | MIC | MBC |
| *B. burgdorferi* #15770 | 0.10 | 0.39 | ≤0.01 | 0.05 | 50 | ≥100 |
| *B. burgdorferi* #2769 | 0.20 | 0.78 | ≤0.01 | 0.02 | 12.5 | ≥100 |
| Range | 0.05-0.39 | 0.20-0.78 | ≤0.01 | 0.02-0.1 | 12.5-50.0 | ≥100 |

microdilution method tended to be higher than those obtained by the macrodilution method, the mean MICs of all three drugs were within one dilution of each other by the two methods. Tilmicosin was the most potent antimicrobial against all *Leptospira* and *Borrelia* isolates on a microgram-per-milliliter basis. All spirochetal isolates were inhibited (MIC) and were killed (MBC) by tilmicosin at below the limit of testing (0.01 µg/ml). The ranges of MIC and MBC of amoxicillin against *Leptospira* spp. were 0.05-6.25 µg/ml and 6.25-25.0 µg/ml, respectively. And the ranges of MIC and MBC of amoxicillin against *B. burgdorferi* were 0.05-0.39 µg/ml and 0.20-0.78 µg/ml, respectively. The ranges of MIC and MBC of enrofloxacin against *Leptospira* spp. were 0.05-0.39 µg/ml and 0.05-0.39 µg/ml, respectively. Two strains of *B. burgdorferi* were resistant to enrofloxacin at the highest concentration tested for MBC (100 µg/ml) (Table 1 and 2).

**Statistical analysis**

Statistical analysis, including all measured MICs and MBCs (n = 144), did not show significant differences in the tested genospecies. The overall reproducibility seen in our study was excellent, although amoxicillin had greater variability than other antimicrobial agents tested.

## Discussion

A uniform and standardized method to test antimicrobial susceptibility against pathogenic spirochetes has not been established. For borreliae and leptospires, the MIC is generally considered the drug concentration at which no motile organisms are observed by dark-field microscopy after 48-72 h of incubation [2,18]. The MBCs for leptospires and borreliae are determined by the absence of spirochetes in subcultures on microscopic examination after various incubation periods [18]. The MBC determinations made by these methods require virtually 100% killing of the final inoculum, which is strict requirement for any antimicrobial agent [9]. In this study, the MBC of antibiotics for leptospires and borreliae was defined as the lowest concentration of the antibiotics at which no spirochetes were subcultured. The macrolide antibiotic tilmicosin produced excellent *in vitro* activity against all 6 strains of spirochetes tested. The MIC and MBC of amoxicillin against *Leptospira* spp. were higher than those of tilmicosin and enrofloxacin, and the

MBC of amoxicillin against *Leptospira* spp. was higher than noted MICs. However, enrofloxacin was not effective against *B. burgdorferi* tested, as shown by its high MIC and MBC.

Tilmicosin is a novel long-acting macrolide antibiotic developed exclusively for veterinary use [28]. As Mycotil, it is used to treat bovine respiratory disease associated with pasteurellae [16,23]. Tilmicosin (10 mg/kg, once) and amoxicillin (15 mg/ml, once or twice) were effective in cattle for resolving experimental leptospirosis caused by *L. borgpetersenii* serovar Hardjo [3,32]. Enrofloxacin was used concurrently with ampicillin in canine leptospirosis and 83% of infected dogs survived [1]. Amoxicillin is effective in treatment of canine Lyme disease [15]. However, there are no data on the clinical use of enrofloxacin or tilmicosin in borreliosis of animals.

Based upon our findings, tilmicosin was superior *in vitro* on a microgram-per-milliliter basis when tested alongside amoxicillin and enrofloxacin under identical test conditions in EMJH or BSK-II media. Moreover, maximum concentrations of tilmicosin in plasma after a subcutaneous dose of 10 mg/kg to cattle are over 100 times higher than the MIC against borreliae and tilmicosin has high volume of distribution that selectively accumulates in solid tissues [22,38]. The accumulation and concentration of tilmicosin in tissues could be due to its lipophilic properties, which favor passage through the lipid membrane of the cells. One mechanism by which the diffusion of tilmicosin may have led to enhance intracellular accumulation is the tendency of basic compounds to be lysosomotropic and become trapped in lysosomes as a result of the acidic pH [30]. The Lyme borrelia reside in the acidic endosome and the use of a lysosomotropic agent augments the clinical activity of macrolide antibiotics in treatment of human patients with chronic borreliosis [11]. Therefore, the potential role of tilmicosin in the treatment of leptospirosis and Lyme disease in some animal species merits further evaluation. Although no significant adverse effects have been reported in cattle, injections of Mycotil to horses, goats, swine, or nonhuman primates can be fatal. Extensive studies on its safety in dogs have not been performed. The heart is the target of toxicity in animals, perhaps mediated via depletion of cardiac intracellular calcium, resulting in a negative inotropic effect [20]. Other formulations of the tilmicosin would be necessary before it could be used in these species.

Classical macrolides and azalides frequently fail in the therapy of early Lyme disease in human and clinical relapse has been observed following conclusion of treatment [14,19, 27,37]. Moreover, it has been speculated that resistance may develop in borreliae preexposed to erythromycin owing to resistant subpopulations. We have evaluated antibiotic treatment using tetracycline, doxcline, and ceftiofur in experimental borreliosis of dogs and horses, however, most of them can not completely eliminate the persistent infection [6,33]. The lack of effective agents in the treatment of animal borreliosis made it urgent to identify other potential antibiotics. Therefore, further evaluation of the potential use of a non-toxic formulation of tilmicosin in the treatment of equine or canine leptopsirosis and/or borreliosis should be confirmed.

## Acknowledgments

## References

1. **Adin CA, Cowgill LD.** Treatment and outcome of dogs with leptospirosis: 36 cases (1990-1998). J Am Vet Med Assoc 2000, **216**, 371-375.

2. **Agger WA, Callister SM, Jobe DA.** In vitro susceptibilities of *Borrelia burgdorferi* to five oral cephalosporins and ceftriaxone. Antimicrob Agents Chemother 1992, **36**, 1788-1790.

3. **Alt DP, Zuerner RL, Bolin CA.** Evaluation of antibiotics for treatment of cattle infected with *Leptospira borgpetersenii* serovar hardjo. J Am Vet Med Assoc 2001, **219**, 636-639.

4. **Appel MJ, Allan S, Jacobson RH, Lauderdale TL, Chang YF, Shin SJ, Thomford JW, Todhunter RJ, Summers BA.** Experimental Lyme disease in dogs produces arthritis and persistent infection. J Infect Dis 1993, **167**, 651-664.

5. **Barbour AG.** Isolation and cultivation of Lyme disease spirochetes. Yale J Biol Med 1984, **57**, 521-525.

6. **Chang YF, Ku YW, Chang CF, Chang CD, McDonough SP, Divers T, Pough M, Torres A.** Antibiotic treatment of experimentally *Borrelia burgdorferi*-infected ponies. Vet Microbiol 2005, **107**, 285-294.

7. **Chang YF, Novosel V, Chang CF, SummersBA, Ma DP, Chiang YW, Acree WM, Chu HJ, Shin S, Lein DH.** Experimental induction of chronic borreliosis in adult dogs exposed to *Borrelia burgdorferi*-infected ticks and treated with dexamethasone. Am J Vet Res 2001, **62**, 1104-1112.

8. **Chang YF, Novosol V, McDonough SP, Chang CF, Jacobson RH, Divers T, Quimby FW, Shin S, Lein DH.** Experimental infection of ponies with *Borrelia burgdorferi*

by exposure to Ixodid ticks. Vet Pathol 2000, **37**, 68-76.

9. **Dever LL, Jorgensen JH, Barbour AG.** In vitro antimicrobial susceptibility testing of *Borrelia burgdorferi*: a microdilution MIC method and time-kill studies. J Clin Microbiol 1992, **30**, 2692-2697.

10. **Divers TJ.** Lyme disease. In: Robinson NE (ed.). Current Therapy in Equine Medicine. 5th ed. pp. 54-56, Saunders, 2003.

11. **Donta ST.** Macrolide therapy of chronic Lyme Disease. Med Sci Monit 2003, **9**, PI 136-142.

12. **Edwards CN, Nicholson GD, Hassell TA, Everard CO, Callender J.** Penicillin therapy in icteric leptospirosis. Am J Trop Med Hyg 1988, **39**, 388-390.

13. **Ellinghausen HC Jr, McCullough WG.** Nutrition of *Leptospira pomona* and growth of 13 other serotypes: fractionation of oleic albumin complex and a medium of bovine albumin and polysorbate 80. Am J Vet Res 1965, **26**, 45-51.

14. **Hansen K, Hovmark A, Lebech AM, Lebech K, Olsson I, Halkier-Sorensen L, Olsson E, Asbrink E.** Roxithromycin in Lyme borreliosis: discrepant results of an in vitro and in vivo animal susceptibility study and a clinical trial in patients with erythema migrans. Acta Derm Venereol 1992, **72**, 297-300.

15. **Hartmann K, Greene CE.** Diseases caused by systemic bacterial infections. In: Ettinger SJ, Feldman EC (eds.). Textbook of Veterinary Internal Medicine: Diseases of the Dog and Cat. 6th ed. pp. 616-631, Elsevier Saunders, St. Louis, 2005.

16. **Laven R, Andrews AH.** Long-acting antibiotic formulations in the treatment of calf pneumonia: a comparative study of tilmicosin and oxytetracycline. Vet Rec 1991, **129**, 109-111.

17. **Levett PN.** Leptospirosis. Clin Microbiol Rev 2001, **14**, 296-326.

18. **Levin JM, Nelson JA, Segreti J, Harrison B, Benson CA, Strle F.** In vitro susceptibility of *Borrelia burgdorferi* to 11 antimicrobial agents. Antimicrob Agents Chemother 1993, **37**, 1444-1446.

19. **Luft BJ, Dattwyler RJ, Johnson RC, Luger SW, Bosler EM, Rahn DW, Masters EJ, Grunwaldt E, Gadgil SD.** Azithromycin compared with amoxicillin in the treatment of erythema migrans. A double-blind, randomized, controlled trial. Ann Intern Med 1996, **124**, 785-791.

20. **Main BW, Means JR, Rinkema LE, Smith WC, Sarazan RD.** Cardiovascular effects of the macrolide antibiotic tilmicosin, administered alone and in combination with propranolol or dobutamine, in conscious unrestrained dogs. J Vet Pharmacol Ther 1996, **19**, 225-232.

21. **McClain JB, Ballou WR, Harrison SM, Steinweg DL.** Doxycycline therapy for leptospirosis. Ann Intern Med 1984, **100**, 696-698.

22. **Modric S, Webb AI, Derendorf H.** Pharmacokinetics and pharmacodynamics of tilmicosin in sheep and cattle. J Vet Pharmacol Ther 1998, **21**, 444-452.

23. **Morck DW, Merrill JK, Gard MS, Olson ME, Nation PN.** Treatment of experimentally induced pneumonic pasteurellosis of young calves with tilmicosin. Can J Vet Res 1997, **61**, 187-192.

24. **Murray CK, Hospenthal DR.** Broth microdilution susceptibility testing for *Leptospira* spp. Antimicrob Agents Chemother 2004, **48**, 1548-1552.

25. **Shryock TR, Apley M, Jones RN, Lein DH, Thornsberry C, Walker JL, White DG, Wu CC.** Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals; approved standard, M31-A2. Vol. 22. pp. 1-80, National Committee for Clinical Laboratory Standards, Wayne, 2002.

26. **Oie S, Hironaga K, Koshiro A, Konishi H, Yoshii Z.** In vitro susceptibilities of five *Leptospira* strains to 16 antimicrobial agents. Antimicrob Agents Chemother 1983, **24**, 905-908.

27. **Oschmann P, Kaiser R.** Therapy and prognosis. In: Oschmann P, Karaiczy P, Halperin J, Brade V (eds.). Lyme Borreliosis and Tick-borne Encephalitis. pp.112-119, UNI-Med Verlag AG, Breman, 1999.

28. **Ose EE.** In vitro antibacterial properties of EL-870, a new semi-synthetic macrolide antibiotic. J Antibiot (Tokyo) 1987, **40**, 190-194.

29. **Panaphut T, Domrongkitchaiporn S, Vibhagool A, Thinkamrop B, Susaengrat W.** Ceftriaxone compared with sodium penicillin G for treatment of severe leptospirosis. Clin Infect Dis 2003, **36**, 1507-1513.

30. **Scorneaux B, Shryock TR.** Intracellular accumulation, subcellular distribution, and efflux of tilmicosin in bovine mammary, blood, and lung cells. J Dairy Sci 1999, **82**, 1202-1212.

31. **Sicklinger M, Wienecke R, Neubert U.** *In vitro* susceptibility testing of four antibiotics against *Borrelia burgdorferi*: a comparison of results for the three genospecies *Borrelia afzelii*, *Borrelia garinii*, and *Borrelia burgdorferi* sensu stricto. J Clin Microbiol 2003, **41**, 1791-1793.

32. **Smith CR, Corney BG, McGowan MR, McClintock CS, Ward W, Ketterer PJ.** Amoxycillin as an alternative to dihydrostreptomycin sulphate for treating cattle infected with *Leptospira borgpetersenii* serovar hardjo. Aust Vet J 1997, **75**, 818-821.

33. **Straubinger RK, Summers BA, Chang YF, Appel,MJ.** Persistence of *Borrelia burgdorferi* in experimentally infected dogs after antibiotic treatment. J Clin Microbiol 1997, **35**, 111-116.

34. **Takashima I, Ngoma M, Hashimoto N.** Antimicrobial effects of a new carboxyquinolone drug, Q-35, on five serogroups of *Leptospira interrogans.* Antimicrob Agents Chemother 1993, **37**, 901-902.

35. **Tayor SM.** Joint disorders. In: Nelson RW, Couto CG (eds.). Small Animal Internal Medicine. 3rd ed. pp. 1079-1092, Mosby, St. Louis, 2003.

36. **Ward MP, Glickman LT, Guptill LE.** Prevalence of and risk factors for leptospirosis among dogs in the United States and Canada: 677 cases (1970-1998). J Am Vet Med Assoc 2002, **220**, 53-58.

37. **Wormser GP, Nadelman RB, Dattwyler RJ, Dennis DT, Shapiro ED, Steere AC, Rush TJ, Rahn DW, Coyle PK, Persing DH, Fish D, Luft BJ.** Practice guidelines for the treatment of Lyme disease. The Infectious Diseases Society of America. Clin Infect Dis 2000, **31 (Suppl 1)**, 1-14.

38. **Ziv G, Shem-Tov M, Glickman A, Winkler M, Saran A.** Tilmicosin antibacterial activity and pharmacokinetics in cows. J Vet Pharmacol Ther 1995, **18**, 340-345.