Indian Journal of Animal Sciences **83** (8): 775–778, August 2013/Article

Susceptibility of *Leptospira* serotypes against various antimicrobials by broth microdilution method

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Received: 20 January 2013; Accepted 6 March 2013

ABSTRACT

In developing countries like India, 90% of patients with leptospirosis have mild flu like symptoms and these patients are treated empirically for fever. Broth microdilution method using resazurin dye reduction assay was followed to test the susceptibility of 30 serovars of *Leptospira* to various antimicrobial drugs that are generally prescribed for fever in India *in vitro*. Among 19 antimicrobial drugs tested against *Leptospira* sp., erythromycin, azithromycin, cefatoxime, penicillin G, ampicillin, amoxycillin and levofloxacin produced MIC_{90} of below the limit of testing i.e.≤0.02 µg/ml. Ceftriaxone, ciprofloxacin and gatifloxacin produced MIC_{90} of 0.05 µg/ml. Cefixime and ofloxacin produced higher MIC_{90} than the other antimicrobial agents tested in cephalosporins and fluoroquinolones group respectively. All tested aminoglycosides, chloramphenicol and tetracycline group of antimicrobials produced higher MIC_{90}. Erythromycin, azithromycin, cefatoxime, penicillin G, ampicillin, amoxicillin and levofloxacin were active against several leptospiral serovars and can be used for treatment of leptospirosis. Broth microdilution method using resazurin dye is simple, convenient and cost effective testing system to assess the susceptibility of leptospiral isolates against a wide variety of antimicrobial drugs in a short duration.

Key words: Antibiotic sensitivity, Broth dilution method, Leptospirosis

Leptospirosis is an anthropozoonosis of ubiquitous distribution, caused by spirochetes of pathogenic *Leptospira* species. The disease is presently endemic and deeply entrenched in South India, Gujarat, Maharashtra and Andaman and Nicobar Islands. Previously, *in vitro* testing of antimicrobial agents for *Leptospira* used a macrobroth dilution methodology which is cumbersome and time consuming (Murgia and Cinco 2001, Prescott 1991). Hospenthal and Murray (2004a and 2004b) developed broth microdilution susceptibility test to detect growth of *Leptospira* and tested 24 antimicrobial agents for 26 *Leptospira* strains. In this study, attempt was made to test 19 antimicrobial agents, which are commonly used in India against 23 reference leptospiral serovars and 7 leptospiral clinical isolates using broth microdilution resazurin dye reduction assay.

MATERIALS AND METHODS

Leptospira strains and isolates: Leptospiral serovars (23) used in this study were obtained from National Reference Laboratory, ICMR, Andaman and Nicobar Islands, and were maintained at Leptospirosis Research Laboratory, Madhavaram Milk Colony, Chennai, India. Other 7 isolates of *Leptospira* were isolated from clinical cases of animal leptospirosis and maintained at Leptospirosis Research Laboratory, Madhavaram Milk Colony, Chennai, India were also used for this study. The details of these serovars represent 23 serogroups and 8 species are presented in the Table 1. All organisms were maintained in EMJH enrichment medium by continuous culture.

Characterization of isolates: A combination of genetic and serologic methods was used to identify isolates. First, the isolates were confirmed as pathogenic leptospires by PCR using E1E2 primers as per Vitale *et al.* (2005). Secondly, random amplification of polymorphic DNA fingerprinting analysis of isolates with reference strains using B11 and B12 was carried out (Gerritsen *et al.* 1995). Finally, antigenic identification (serogroup) of isolates was done by microscopic agglutination test using reference serum obtained from Pasteur Institute, France.

Antimicrobial agents: The following antibiotics were evaluated in this study: Penicillin G, ampicillin, amoxycillin, cefotaxime, ceftriaxone, streptomycin, gentamicin, amikacin, chloramphenicol, erythromycin, tetracycline,

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Table 1. *Leptospira* strains used for antimicrobial susceptibility testing

Species	Serogroup	Serovar	Strain
L.interrogans	*Australis*	*Australis*	*Ballico*
L.interrogans	*Autumnalis*	*Bankinang I*	*Akiyami A*
L.interrogans	*Canicola*	*Canicola*	*Hond Utrecht IV*
L.interrogans	*Icterohaem-orrhagiae*	*Icterohaem-orrhagiae*	*RGA*
L.interrogans	*Hebdomadis*	*Hebdomadis*	*Hebdomadis*
L.interrogans	*Pomona*	*Pomona*	*Pomona*
L.interrogans	*Pyrogenes*	*Pyrogenes*	*Salinem*
L.interrogans	Bataviae	Bataviae	*Swart*
L.interrogans	Djasiman	Djasiman	Djasiman
L.interrogans	*Sejroe*	*Hardjo*	*Hardjoprajitno*
L.borgpetersenii	*Ballum*	*Ballum*	*Mus 127*
L.borgpetersenii	*Mini*	*Mini*	*Sari*
L.borgpetersenii	*Tarassovi*	*Tarassovi*	*Perepelicin*
L.noguchii	*Louisiana*	*Louisiana*	*LSU 1945*
L.noguchii	*Panama*	*Panama*	*CZ 214 K*
L.weilii	*Celledoni*	*Celledoni*	*Celledoni*
L.weilii	*Sarmin*	*Sarmin*	*Sarmin*
L.kirschneri	*Cynopteri*	*Cynopteri*	*3522 C*
L.kirschneri	*Grippotyphosa*	*Grippotyphosa*	*Moskva V*
L.santarosai	*Manhao*	*Manhao*	*L 60*
L.santarosai	*Shermani*	*Shermani*	*1342 K*
L.fainei	*Hurstbridge*	*Hurstbridge*	*BUT 6*
L.biflexa	*Semaranga*	*Patoc*	*Patoc I*
L.interrogans	*Australis*	*Australis*	
L.interrogans	*Australis*	*Australis*	
L.interrogans	*Australis*	*Australis*	
L.interrogans	*Hebdomadis*	*Hebdomadis*	
L.interrogans	*Hebdomadis*	*Hebdomadis*	
L.interrogans	*Canicola*	*Canicola*	
L.borgpetersenii	*Ballum*	*Ballum*	

oxytetracycline, doxycycline, ciprofloxacin, ofloxacin, levofloxacin, gatifloxacin, azithromycin and cefixime. Stock solution of antibiotics were prepared as 1 mg/ml concentration in EMJH media and filtered through 0.2 µm and stored at –20°C in aliquots.

Determination of minimum inhibitory concentrations: The MIC was determined by using broth microdilution method as per Hospenthal and Murray (2004a) with modification. Final drug concentration of each antibiotic was from 25 µg/ml to 0.02 µg/ml (units per Litre for penicillin G), in a total volume of 50 µl per well. On the day 4, MIC was calculated as the lowest concentration at which there was no blue-to-pink colour change. Each strain-drug combination was tested in triplicate and median MIC was used for comparisons. Cumulative susceptibility results across all serovars have been expressed as MIC_{90}, the concentration at which 90% of the *Leptospira* isolates were inhibited.

RESULTS AND DISCUSSION

Leptospirosis is primarily a contagious disease of animals, occasionally infecting humans. The change in the distribution and incidence rate of leptospirosis has occurred proportionately to the alterations in the eco-system. Almost 90% of anicteric leptospirosis patients/cases have fever, headache and myalgia. Hence, they are initially treated empirically due to the lack of rapid and accurate diagnostic testing. This necessitates therapeutic decisions that may cover a broad range of infectious diseases as part of the differential diagnosis.

Currently, there is no standard method to assess antimicrobial agents for antileptospiral activity due to the inability of the leptospires to grow in solid media. Studies involving *in vivo* testing of antimicrobial agents in humans and even in livestock are very limited (Alt and Bolin 1996; Panaphut *et al*. 2003). Earlier broth macrodilution method was employed which is laborious, time consuming and not cost effective. Hospenthal and Murray (2004a) have developed a rapid, simple, reliable broth microdilution method for testing susceptibility of organisms of the genus *Leptospira* to antimicrobial agents. In this study, microbroth dilution method using resazurin dye was also used to test isolates and reference strains against commonly prescribed drugs for fever in India.

The MIC_{50} and MIC_{90} using the serovars against 19 antimicrobial drugs are reported in Table 2. Penicillin G, ampicillin, amoxicillin, cefotaxime, erythromycin, azithromycin, levofloxacin were all found to have MIC_{90} below the limit of testing (<0.02 µg/ml). The MIC_{90} of ceftriaxone, gatifloxacin, ciprofloxacin were ≤0.05 µg/ml for all serovars tested in triplicates. The MIC_{90} of oflaxacin was ≤0.2 µg/ml, oxytetracycline was ≤0.39 µg/ml, tetracycline and cefixime were ≤0.78 µg/ml. Amikacin, streptomycin, gentamicin and chloramphenicol all produced the MIC_{90}s

Table 2. Minimum inhibitory concentration of antimicrobial drugs against 30 *Leptospira* serovars

Antimicrobial drugs	MIC_{50}	MIC_{90}
Tetracycline	0.2	0.78
Oxytetracycline	0.1	0.39
Doxycycline	3.13	6.25
Penicillin G	≤0.02	≤0.02
Ampicillin	≤0.02	≤0.02
Amoxycillin	≤0.02	≤0.02
Cefixime	0.2	0.78
Cefotaxime	≤0.02	≤0.02
Ceftriaxone	≤0.02	0.05
Streptomycin	0.2	1.56
Gentamicin	0.78	3.13
Amikacin	0.78	1.56
Erythromycin	≤0.02	≤0.02
Azithromycin	≤0.02	≤0.02
Ciprofloxacin	≤0.02	0.05
Gatifloxacin	≤0.02	0.05
Levofloxacin	≤0.02	≤0.02
Ofloxacin	0.1	0.2
Chloramphenicol	0.39	3.13

of ≤3.13 µg/ml. Surprisingly, MIC_{90} of doxycycline was 6.25 µg/ml.

Hospenthal and Murray (2004b) determined susceptibility of 26 leptospiral serovars to 24 antimicrobial agents. To compare, 12 serovars and 13 antimicrobials were overlapping in both studies. Among them, 78 out of 156 (exactly 50%) test results were well correlated within 2 dilutions. The correlation increases to 89% if tetracycline, doxycycline, penicillin, ampicillin amoxicillin and chloramphenicol are excluded from this comparison. The test results were 100% correlated for azithromycin and erythromycin, 92% for gatifloxacin and 83% for cefatoxime. The minor dilution variation between 2 systems for the same serovar to the same antimicrobial agent may be due to the use of different concentration of resazurin dye in the studies and non-uniform growth rates of the same strain in different laboratory conditions. Remarkably, higher MIC_{90} was also reported for tetracycline, doxycyline and chloramphenicol by Hospenthal and Murray (2004 a, b) corroborate well with our results.

Oxytetracycline had the lowest MIC_{90} (0.39 µg/ml) of the 3 tetracycline group of antimicrobial agents followed by tetracycline (0.78 µg/ml) and doxycycline (6.25 µg/ml) against many of the tested leptospires. The aminoglycosides exhibited higher MIC_{90} for all tested serovar of *Leptospira*. Amikacin and Streptomycin had MIC_{90} of 1.56 µg/ml and gentamicin had little higher MIC_{90} of 3.13 µg/ml. Oie *et al.* (1983) reported higher MIC for 4 kinds of aminoglycosides against 5 *Leptospira* serovars tested by tube dilution method.

The betalactam group of antibiotics showed greater activity against all *Leptospira* serovars indicating their clinical utility in leptospirosis. Hospenthal and Murray (2004a, b) reported that penicillin G, ampicillin and amoxycillin produced higher MIC_{90s} to all serovars tested contradictory toour result. However, Ressner *et al.* (2008) from the same laboratory have reported increased activities of ampicillin and penicillin G against virulent strain compared to their activities against the laboratory-passaged strain.

Cefixime produced the highest MIC_{90} (0.78 µg/ml) of the 3 kinds of cephalosporin antibiotics tested. Although there is no available literature for antimicrobial susceptibility testing of cefixime against leptospires, it exhibited weaker bactericidal activity than ceftriaxone and cefotaxime against another spirochete *Borrelia burgdorferi* (Hunfeld *et al*. 2000, 2003). Ceftriaxone had little higher MIC_{90} (0.05 µg/ml) than cefatoxime (≤0.02 µg/ml). Ressner *et al*. (2008) and Hospenthal and Murray (2004b) also reported the higher activity of cefatoxime over ceftriaxone for leptospires tested in microbroth dilution method.

Both *in vivo* and *in vitro* studies proved consistently that erythromycin, azithromycin and cefotaxime were more effective against all leptospires and they could be ideal for the treatment of acute leptospirosis (Griffith *et al*. 2007, Moon *et al*. 2007). However, cefotaxime has to be administered parentally and erythromycin cannot be used during pregnancy. Considering above facts along with the *in vivo* study by Moon *et al.* (2007), azithromycin may be used as an alternative option for doxycycline as a prophylaxis as well as for treatment of leptospirosis.

Wuthiekanun *et al*. (2013) developed a new agar LVW that facilitate the growth of leptospiral sp. in solid agar media and also used the media to develop E test to determine the MICs of leptospiral isolates against 5 commonly used drugs against leptospirosis. They have compared E test and microbroth dilution method for the above 5 drugs and of 15 MICs, 7 (47%) were the same for both methods, 5 (33%) differed by no more than 2 dilutions, and 3 (20%) were different by 3 dilutions.

Earlier broth macrodilution method has been employed which is laborious and not cost effective. Resazurin dye is an oxidation-reduction indicator which is used for the evaluation of cell growth, particularly in various cytotoxicity assays (McNicoll *et al*. 2007). It is a blue non-fluorescent dye that becomes pink and fluorescent when reduced to resarufin by oxidoreductases within viable cells. Resazurin is less toxic to cells as well as safe for users and it will not pose any threat to environment on disposals. A resazurin dye is readily available in developing countries since resazurin reduction test has also been used for decades to demonstrate bacterial and yeast contamination of milk. Hence, we have used resazurin dye for detecting cell viability. Broth microdilution method using resazurin dye is simple, reproducible, convenient, time saving and cost effective testing system to assess the susceptibility of leptospiral isolates against a wide variety of antimicrobial drugs in a short duration. This broth microdilution method can also be applied to study the susceptibility of other slow growing organisms like *M. tuberculosis* and *M. avium* subsp. *paratuberculosis*.

ACKNOWLEDGEMENTS

Author is grateful to Dr V. Jayakumar and Dr B Murali Manohar for their logistic and administrative help and is also thankful to Mr V Baskar and Ms M Indumathy for their assistance in carrying out this research.

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