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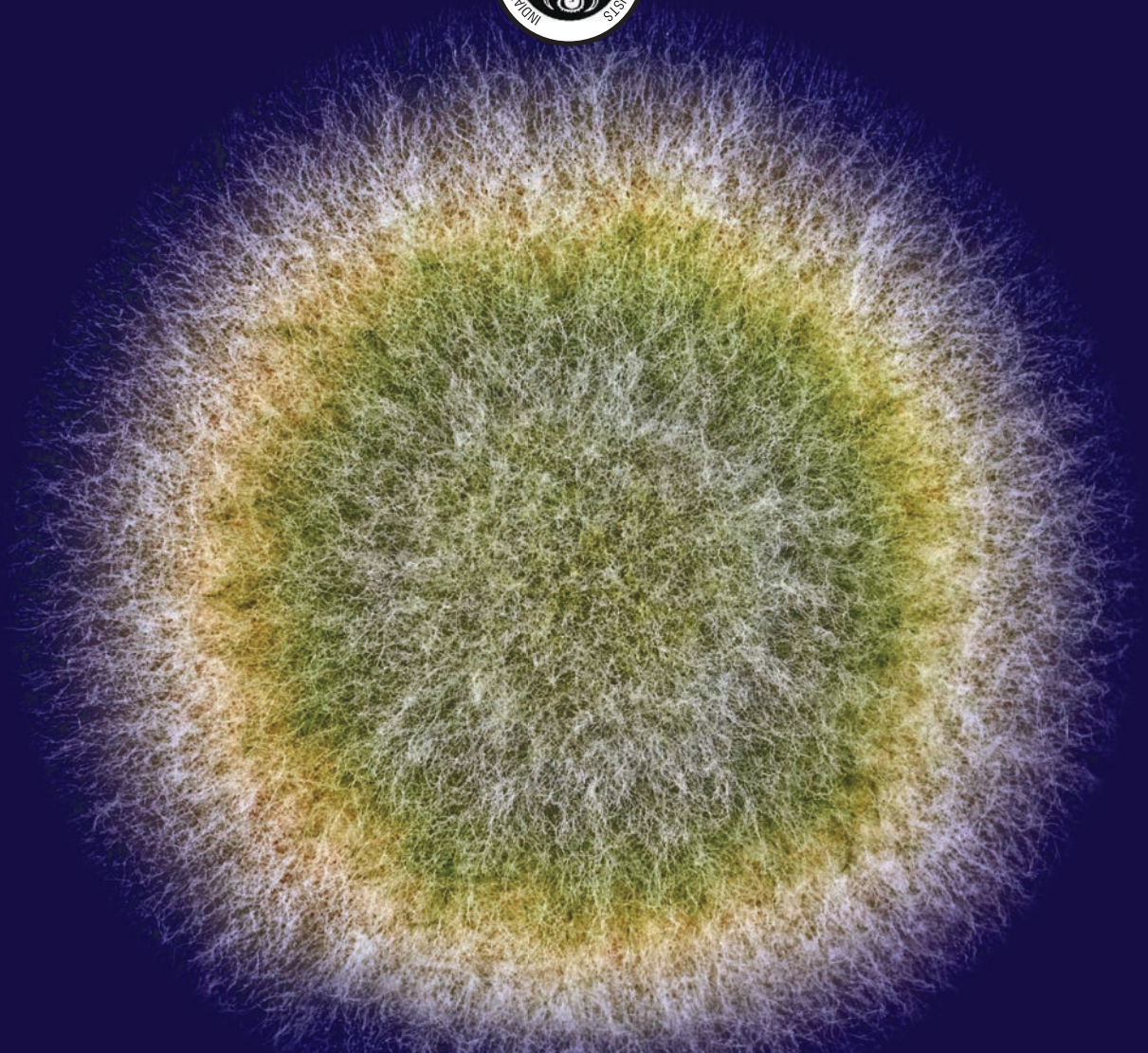

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Brief Communication

Antimicrobial Susceptibility of *Leptospira* spp. Isolated from Environmental, Human and Animal Sources in Malaysia

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Abstract

Leptospirosis is a zoonosis with worldwide distribution caused by pathogenic spirochetes of the genus *Leptospira*. The aim of this study was to evaluate the susceptibility of isolates obtained from different hosts. A total of 65 *Leptospira* isolates from humans (n = 1), zoonoses (rat, n = 60; dog, n = 1; swine, n = 1) and environment (n = 2) were tested against six antibiotics. All the isolates were resistant to trimethoprim and sulphamethoxazole and had high MIC toward chloramphenicol (MIC_{90}: 6.25 μg/ml). All except one environment isolate were sensitive to ampicillin, doxycycline and penicillin G.

Keywords: Antimicrobial susceptibility, *Leptospira*, leptospirosis, minimal inhibitory concentration

Introduction

Leptospirosis is an emerging zoonotic disease with worldwide distribution.[1] It is caused by pathogenic spirochetes of the genus *Leptospira*. Approximately, one-half of the pathogenic serovars belong to *Leptospira interrogans* or *Leptospira borgpetersenii*.[2] Rodents are the principal known maintenance hosts, besides domestic animals, livestock and wild animals.[2] Humans become accidental hosts by acquiring the infection through direct contact with urine, blood or infected animal tissue or indirect contact with water or soil contaminated with the urine from reservoir animals.[1] Clinically, symptoms of infection may range in severity from mild to fatal, depending on the infection stage. However, the clinical presentation of leptospirosis is unspecific, which frequently leads to misdiagnosis.[3] In Malaysia, misdiagnosis of this infection has become a critical issue, where dengue, malaria and other infectious diseases with overlapping clinical presentations are endemic. Treatment normally follows an empirical chemotherapy route, which requires information regarding the susceptibilities of *Leptospira* isolates to various antimicrobial agents. Effective and appropriate antibiotic selection for treatment is essential to prevent complications. Several studies have been carried out on *Leptospira* isolates' susceptibilities. However, these studies have a number of limitations, such as using laboratory-passaged strains or a small number of *Leptospira* strains.[4] Due to the endemicity of leptospirosis in Malaysia[5] as well as the dramatic increase in reported cases over the last decade, there is a critical need to determine the effectiveness of common antibiotics in controlling this organism. However, no information is available on the antimicrobial susceptibilities of Malaysian *Leptospira* isolates. Therefore, the aim of this study is to monitor and evaluate the susceptibility of recent local isolates obtained from different hosts and sites to traditional antimicrobial compounds used for leptospirosis treatment.

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Materials and Methods

Bacterial strains

Sixty-five *Leptospira* isolates representing four different species and at least five serovars were included in the testing. These isolates from environmental (water, n = 2) and animal sources (rat, n = 60; dog, n = 1; swine, n = 1) were isolated between 2011 and 2014 from different sites in six states in Peninsular Malaysia.[2,5] One clinical isolate was provided by

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the Royal Tropical Institute (KIT), Amsterdam, Netherlands. The strain was originally isolated from a human in Malaysia by Alexander *et al*.[6] The isolates were maintained in culture in Ellinghausen-McCullough-Johnson-Harris (EMJH) medium. Information on the isolates is summarised in Table 1.

Antimicrobial agents

The antimicrobial solutions employed in this study were prepared from reagent grade powders to produce 1 mg/ml solutions using solvents and diluents suggested in the Clinical and Laboratory Standards Institute document M100-S22[7] or according to the manufacturer's suggestions if available. Six antimicrobial agents, including doxycycline, penicillin G, trimethoprim, ampicillin, chloramphenicol (MP Biomedicals, France) and sulphamethaxazole (Sigma-Aldrich; St. Louis, MO, USA) were used to test the *Leptospira* isolates' antimicrobial susceptibility. The stock antimicrobial solutions were stored at −80°C in divided one-time use aliquots.

Susceptibility testing

Broth microdilution testing was performed as reported by Murray and Hospenthal[4] and Chakraborty *et al*.[8] The antibiotic concentrations ranged from 25.0 to 0.01 μg/ml (units/ml for penicillin), with the exception of sulphamethaxazole and trimethoprim, which were tested at 3200 to 12.5 μg/ml. The strain *L. interrogans* serovar Icterohaemorrhagiae was used for internal validation with minimal inhibitory concentration (MIC) parameters and served as the control strain.[4] All tests were performed in triplicate and included positive (bacteria only) and negative controls (medium only). The *Leptospira* inoculum used for testing was prepared from 7-day-old cultures grown in liquid EMJH medium at 30°C. Then, 100 μl *Leptospira* inoculum of 2×10^6 leptospiral organisms/ml was added, and the 96 well plates were incubated at 30°C, with each well having a final volume of 200 μl. Following 3 days of incubation, 20 μl of 10X alamarBlue (Invitrogen, Cleveland, OH, USA) was added to each well. AlamarBlue is an oxidation-reduction indicator that changes colour from dark blue to bright pink in response to the chemical reduction of the growth medium resulting from cell development. The colour of each well was documented on the 5th day of incubation, and the MICs were recorded as the concentration in the well containing the lowest concentration without blue-to-pink colour change.

Results

The MIC_{90} values are reported in Table 1. All isolates were resistant to trimethoprim and sulphamethoxazole (MIC_{90}: 1600 μg/ml). All isolates had an MIC_{90} range of 3.13–6.25 μg/ml to chloramphenicol, except that from swine (LS01/11) and one isolate from rat (LR31/13) which showed higher MICs of 12.5 and 25 μg/ml, respectively. Overall, penicillin G and ampicillin appeared to be effective for all clinical and zoonotic isolates with MIC_{90} between 0.1 and 0.2 μg/ml. Only the dog isolate showed a slightly higher MIC toward penicillin G (0.39 μg/ml) compare to the rest while the lowest was toward the swine isolate (<0.01 μg/ml). However, the environmental isolate *L. kirschneri* (LE02/11) was resistant to penicillin G and ampicillin as the MIC reported was 25 μg/ml. The MICs of doxycycline ranged from 0.2 to 0.78 μg/ml, but one zoonotic (LS01/11) and 2 environmental isolates (LE01/11, LE02/11) displayed a higher MIC toward doxycycline (3.13 μg/ml) compared to the other isolates although still remained susceptible. Overall, doxycycline was more effective on all isolates tested compared to ampicillin and penicillin G.

Discussion

Leptospirosis is an endemic disease in Malaysia, occurring in both urban and rural locations. The local absence of sensitive, specific and rapid methods of diagnosing leptospirosis makes it difficult to distinguish it from other febrile illnesses. The misdiagnosis of leptospirosis often leads to treatment with a broad range of antimicrobials that cover the febrile syndromes of various local illnesses. Therefore, this study was carried out to monitor and determine the susceptibility patterns in the different isolates of leptospirosis reflecting on their effectiveness in the treatment of leptospirosis.

In our study, both trimethoprim and sulphamethoxazole were found ineffective against all isolates tested. This finding is in agreement with previous studies.[8,9] Trimethoprim and sulphamethoxazole have sometimes been applied in combination for their synergistic action in providing a broad-spectrum bactericidal antimicrobial coverage before definitive diagnosis.[10] However, the resistance of *Leptospira* strains to these antibiotics may compel health workers to consider other antileptospiral drugs in cases where the diagnosis of leptospirosis is inconclusive.

The local isolates demonstrated a higher MIC toward chloramphenicol, which is in agreement with the findings of Murray and Hospenthal.[4] Previously, chloramphenicol appeared to be effective against *Leptospira* in experimental mice.[11] However, a higher concentration was required to produce an inhibition or a bactericidal effect on *Leptospira* strains.[4] Unlike chloramphenicol, ampicillin displayed a lower MIC to the isolates tested, except for one environmental isolate that was resistant (MIC: 25 μg/ml). Administration of ampicillin was a potential option in the treatment of this illness both *in vivo* and *in vitro*.[8,12,13] However, the action of ampicillin against leptospirosis is restricted and cannot be distributed to all organ tissues, such as the kidneys and the heart, rendering it ineffective in clearing leptospires located in protected sites.[13] Currently, penicillin G and doxycycline are recognised as ideal drugs for the treatment of leptospirosis.[9] The MIC results of penicillin G were similar to those produced by ampicillin. However, penicillin G is generally recommended for treating severe leptospirosis. The advantages of using penicillin G include low toxicity and the potential to administer the drug intramuscularly or intravenously at high doses in the early stages of infection.[14]

Table 1: Strains of *Leptospira* tested and their susceptibility to six antimicrobial agents

Strains number	Species/serovar	Isolation site	Isolation source	MIC (μg/ml)[a]					
				PenG	AMP	CAM	DOXY	SMX	TMP
LR01/11	*L. borgpetersenii*/Javanica	Kuala Lumpur	Rat	0.2	0.05	3.13	0.39	800	800
LR02/11	*L. borgpetersenii*/Javanica	Kuala Lumpur	Rat	0.2	0.1	6.25	0.39	1600	1600
LR03/11	*L. borgpetersenii*/Javanica	Kuala Lumpur	Rat	0.2	0.1	6.25	0.39	1600	1600
LR04/11	*L. borgpetersenii*/Javanica	Kuala Lumpur	Rat	0.2	0.1	6.25	0.39	1600	1600
LR05/11	*L. borgpetersenii*/Javanica	Kuala Lumpur	Rat	0.2	0.1	6.25	1.56	1600	1600
LR06/11	*L. borgpetersenii*/Javanica	Kuala Lumpur	Rat	0.2	0.1	6.25	0.39	1600	1600
LR07/11	*L. borgpetersenii*/Javanica	Kuala Lumpur	Rat	0.2	0.1	6.25	0.39	1600	1600
LR08/11	*L. borgpetersenii*/Javanica	Kuala Lumpur	Rat	0.2	0.1	6.25	0.39	1600	1600
LR09/11	*L. interrogans*/Bataviae	Kuala Lumpur	Rat	>0.2	0.1	6.25	>0.2	1600	1600
LR10/11	*L. borgpetersenii*/Javanica	Kuala Lumpur	Rat	0.2	0.1	6.25	0.39	1600	1600
LR11/11	*L. borgpetersenii*/Javanica	Kuala Lumpur	Rat	0.02	0.05	6.25	0.39	800	1600
LR12/11	*L. interrogans*/Bataviae	Kuala Lumpur	Rat	>0.2	0.1	6.25	>0.2	1600	1600
LR13/11	*L. interrogans*/Bataviae	Penang	Rat	>0.2	0.1	6.25	>0.2	1600	1600
LR14/11	*L. borgpetersenii*/Javanica	Penang	Rat	0.2	0.05	6.25	0.39	1600	1600
LR15/11	*L. interrogans*/Bataviae	Penang	Rat	0.2	0.1	6.25	0.39	1600	800
LR16/11	*L. interrogans*/Bataviae	Penang	Rat	0.2	0.1	6.25	0.39	1600	800
LR17/11	*L. interrogans*/Bataviae	Penang	Rat	0.2	0.1	6.25	0.39	1600	800
LR18/12	*L. borgpetersenii*/Javanica	Kuala Lumpur	Rat	0.2	0.1	6.25	0.39	1600	1600
LR19/12	*L. interrogans*/unknown	Kuala Lumpur	Rat	0.2	0.05	3.13	0.39	800	800
LR20/12	*L. borgpetersenii*/Javanica	Kuala Lumpur	Rat	0.2	0.1	6.25	0.39	1600	1600
LR21/12	*L. borgpetersenii*/Javanica	Kuala Lumpur	Rat	0.2	0.1	6.25	0.39	1600	1600
LR22/12	*L. borgpetersenii*/Javanica	Kuala Lumpur	Rat	0.2	0.1	6.25	0.39	1600	1600
LR23/12	*L. borgpetersenii*/Javanica	Kuala Lumpur	Rat	0.2	0.1	6.25	0.39	1600	1600
LR24/12	*L. borgpetersenii*/Javanica	Kuala Lumpur	Rat	0.2	0.1	6.25	0.39	1600	1600
LR25/12	*L. borgpetersenii*/Javanica	Kuala Lumpur	Rat	0.2	0.1	6.25	0.39	1600	1600
LR26/12	*L. interrogans*/Bataviae	Kuala Lumpur	Rat	0.2	0.1	6.25	0.39	1600	800
LR27/13	*L. borgpetersenii*/Javanica	Ipoh	Rat	0.1	0.05	6.25	0.39	1600	1600
LR28/13	*L. borgpetersenii*/Javanica	Ipoh	Rat	0.2	0.05	6.25	0.39	1600	1600
LR29/13	*L. borgpetersenii*/Javanica	Ipoh	Rat	0.1	0.2	25	0.78	>3200	3200
LR30/13	*L. borgpetersenii*/Javanica	Ipoh	Rat	0.2	0.1	6.25	0.39	>1600	1600
LR31/13	*L. borgpetersenii*/Javanica	Ipoh	Rat	0.2	0.1	6.25	0.39	>1600	1600
LR32/13	*L. borgpetersenii*/Javanica	Ipoh	Rat	0.1	0.05	6.25	0.39	1600	1600
LR33/13	*L. borgpetersenii*/Javanica	Ipoh	Rat	0.2	0.1	6.25	0.39	1600	1600
LR34/13	*L. borgpetersenii*/Javanica	Ipoh	Rat	0.1	0.05	6.25	0.39	3200	1600
LR35/13	*L. borgpetersenii*/Javanica	Ipoh	Rat	0.1	0.05	6.25	0.39	>1600	1600
LR36/13	*L. borgpetersenii*/Javanica	Ipoh	Rat	0.2	0.05	6.25	0.39	1600	1600

Contd...

Table 1: Contd...

Strains number	Species/serovar	Isolation site	Isolation source	MIC (μg/ml)[a]					
				PenG	AMP	CAM	DOXY	SMX	TMP
LR37/13	*L. borgpetersenii*/Javanica	Ipoh	Rat	0.2	0.05	6.25	0.39	1600	1600
LR38/13	*L. borgpetersenii*/Javanica	Ipoh	Rat	0.2	0.1	6.25	0.78	1600	1600
LR39/13	*L. borgpetersenii*/Javanica	Ipoh	Rat	0.2	0.05	6.25	0.39	1600	1600
LR40/13	*L. interrogans*/Bataviae	Malacca	Rat	0.2	0.05	6.25	0.39	1600	1600
LR41/13	*L. interrogans*/Bataviae	Malacca	Rat	0.2	0.05	6.25	0.39	1600	1600
LR42/13	*L. interrogans*/Bataviae	Malacca	Rat	0.2	0.05	6.25	0.39	1600	1600
LR43/13	*L. interrogans*/Bataviae	Malacca	Rat	0.2	0.05	6.25	0.39	1600	1600
LR44/13	*L. interrogans*/Bataviae	Malacca	Rat	0.2	0.05	6.25	0.39	1600	1600
LR45/13	*L. interrogans*/Bataviae	Malacca	Rat	0.2	0.05	6.25	0.39	1600	1600
LR46/13	*L. borgpetersenii*/Javanica	Ampang	Rat	0.2	0.1	6.25	0.39	1600	1600
LR47/13	*L. interrogans*/Bataviae	Ampang	Rat	0.1	0.05	6.25	0.39	3200	1600
LR48/13	*L. interrogans*/Bataviae	Ampang	Rat	0.1	0.05	6.25	0.39	3200	1600
LR49/14	*L. interrogans*/Bataviae	Kuantan	Rat	0.1	0.05	6.25	0.39	3200	1600
LR50/14	*L. interrogans*/Bataviae	Kuantan	Rat	0.1	0.05	6.25	0.39	3200	1600
LR51/14	*L. interrogans*/Bataviae	Kuantan	Rat	0.2	0.05	6.25	0.39	1600	1600
LR52/14	*L. interrogans*/Bataviae	Kuantan	Rat	0.2	0.05	6.25	0.39	1600	1600
LR53/14	*L. interrogans*/Bataviae	Kuantan	Rat	0.2	0.05	6.25	0.39	1600	1600
LR54/14	*L. interrogans*/Bataviae	Kuantan	Rat	0.2	0.05	6.25	0.39	1600	1600
LR55/14	*L. interrogans*/Bataviae	Kuantan	Rat	0.2	0.05	6.25	0.39	1600	1600
LR56/14	*L. interrogans*/Bataviae	Kuantan	Rat	0.2	0.05	6.25	0.39	1600	1600
LR57/14	*L. interrogans*/Bataviae	Kuantan	Rat	0.2	0.05	6.25	0.39	1600	1600
LR58/14	*L. borgpetersenii*/Javanica	Kuantan	Rat	0.2	0.1	6.25	0.39	1600	1600
LR59/14	*L. interrogans*/Bataviae	Kuantan	Rat	>0.2	0.1	6.25	>0.2	1600	1600
LR60/14	*L. interrogans*/Bataviae	Kuantan	Rat	>0.2	0.1	6.25	>0.2	1600	1600
LD01/11	*L. interrogans*/Canicola	Kuala Lumpur	Dog	0.39	0.2	6.25	0.39	1600	1600
LS01/11	*L. interrogans*/Pomona	Selangor	Swine	≤0.01	0.02	12.5	3.13	1600	800
LH01/57	*L. interrogans*/Ricardi	Pahang	Human	0.2	0.1	3.13	0.78	800	800
LE01/11	*L. kmetyi*	Ipoh	Water	0.2	0.1	6.25	3.13	800	800
LE02/11	*L. kirschneri*	Ipoh	Water	25	25	6.25	3.13	400	800
MIC_{90}				>0.2	0.1	6.25	0.78	1600	1600

[a]Values for penicillin are in units/ml; all others are in μg/ml. MIC_{90}: The concentration at which 90% of the *Leptospira* isolates are inhibited.
PenG: Penicillin G, AMP: Ampicillin, CAM: Chloramphenicol, DOXY: Doxycycline, SMX: Sulphamethoxazole, TMP: Trimethoprim, *L. borgpetersenii*: *Leptospira borgpetersenii*, *L. interrogans*: *Leptospira interrogans*, *L. kmetyi*: *Leptospira kmetyi*, *L. kirschneri*: *Leptospira kirschneri*, MIC: Minimal inhibitory concentration

The efficacy of penicillin was compared with ceftriaxone in a trial study in treatment of severe leptospirosis, where one group ($n = 86$) was given intravenous penicillin G 1.5 million unit/6 h and second group ($n = 87$) was given intravenous ceftriaxone 1 g daily for 7 days. After 7 days follow-up, no significant difference was observed for median duration of fever, mortality and complications such as renal failure, jaundice and thrombocytopenia.[15]

In this study, one environmental and one zoonotic isolate showed a slightly higher MIC with doxycycline than the rest of the isolates. However, this antibiotic was still effective on all isolates – a finding which correlated with previous studies.[8,16] Doxycycline has been widely recommended and utilised for the prophylaxis and treatment of mild leptospirosis.[17] In the leptospirosis outbreak that occurred in an eco-challenge multisport competition in Sabah, Malaysia, athletes who had taken doxycycline before the challenge were spared from infection.[18] Truccolo *et al*.[13] used quantitative PCR assay to evaluate ampicillin, ofloxacin and doxycycline for treatment of experimental leptospirosis. The results showed the ability of ampicillin at a high dose (100 mg/kg of body weight) to clear leptospires from the host, except from kidneys and heart, where 10^2 leptospires/g remained at day 6. Ofloxacin (30 mg/kg) was unable to clear bacteria from blood or kidneys. With doxycycline (10 mg/kg), the clearance of leptospires occurred in 2 days in all the target organs studied, with the exception of liver, which required 3 days. They concluded that doxycycline had the potential for the treatment of leptospirosis cases compared to other two antibiotics used.

The environmental isolate *L. kirschneri* had higher MIC with the antibiotics tested compared to clinical and zoonotic isolates. In a study performed by Murray and Hospenthal,[7] the results indicated that *L. kirschneri* had a higher MIC toward some of the antibiotics tested compared to other species.

Conclusion

Doxycycline, ampicillin and penicillin G are still effective against all clinical and zoonotic isolates. However, further testing on larger numbers of environmental isolates is required to determine the most suitable antibiotic treatment for leptospirosis.

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Nil.

Conflicts of interest

There are no conflicts of interest.

References

1. Bharti AR, Nally JE, Ricaldi JN, Matthias MA, Diaz MM, Lovett MA, *et al.* Leptospirosis: A zoonotic disease of global importance. Lancet Infect Dis 2003;3:757-71.
2. Benacer D, Mohd Zain SN, Amran F, Galloway RL, Thong KL. Isolation and molecular characterization of *Leptospira interrogans* and *Leptospira borgpetersenii* isolates from the urban rat populations of Kuala Lumpur, Malaysia. Am J Trop Med Hyg 2013;88:704-9.
3. Bruce MG, Sanders EJ, Leake JA, Zaidel O, Bragg SL, Aye T, *et al.* Leptospirosis among patients presenting with dengue-like illness in Puerto Rico. Acta Trop 2005;96:36-46.
4. Murray CK, Hospenthal DR. Broth microdilution susceptibility testing for *Leptospira* spp. Antimicrob Agents Chemother 2004;48:1548-52.
5. Benacer D, Woh PY, Mohd Zain SN, Amran F, Thong KL. Pathogenic and saprophytic *Leptospira* species in water and soils from selected urban sites in peninsular Malaysia. Microbes Environ 2013;28:135-40.
6. Alexander AD, Evans LB, Toussaint AJ, Marchwicki RH, Mccrumb FR Jr. Leptospirosis in Malaya. II. Antigenic analysis of 110 Leptospiral strains and other serologic studies. Am J Trop Med Hyg 1957;6:871-89.
7. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing. 22nd Informational Supplement. CLSI Document M100-S22. Wayne, Pennsylvania, USA: Clinical and Laboratory Standards Institute; 2012.
8. Chakraborty A, Miyahara S, Villanueva SY, Gloriani NG, Yoshida S. *In vitro* sensitivity and resistance of 46 *Leptospira* strains isolated from rats in the Philippines to 14 antimicrobial agents. Antimicrob Agents Chemother 2010;54:5403-5.
9. Miraglia F, Matsuo M, Morais ZM, Dellagostin OA, Seixas FK, Freitas JC, *et al.* Molecular characterization, serotyping, and antibiotic susceptibility profile of *Leptospira interrogans* serovar Copenhageni isolates from Brazil. Diagn Microbiol Infect Dis 2013;77:195-9.
10. Chetchotisakd P, Chierakul W, Chaowagul W, Anunnatsiri S, Phimda K, Mootsikapun P, *et al.* Trimethoprim-sulfamethoxazole versus trimethoprim-sulfamethoxazole plus doxycycline as oral eradicative treatment for melioidosis (MERTH): A multicentre, double-blind, non-inferiority, randomised controlled trial. Lancet 2014;383:807-14.
11. Dunn MC, Thompson PE. Chemotherapy of experimental leptospirosis with chloramphenicol, (chloromycetin), subtilin and penicillin G. J Infect Dis 1953;92:33-9.
12. Alt DP, Bolin CA. Preliminary evaluation of antimicrobial agents for treatment of *Leptospira interrogans* serovar pomona infection in hamsters and swine. Am J Vet Res 1996;57:59-62.
13. Truccolo J, Charavay F, Merien F, Perolat P. Quantitative PCR assay to evaluate ampicillin, ofloxacin, and doxycycline for treatment of experimental leptospirosis. Antimicrob Agents Chemother 2002;46:848-53.
14. Watt G, Padre LP, Tuazon ML, Calubaquib C, Santiago E, Ranoa CP, *et al.* Placebo-controlled trial of intravenous penicillin for severe and late leptospirosis. Lancet 1988;1:433-5.
15. 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;36:1507-13.
16. Ressner RA, Griffith ME, Beckius ML, Pimentel G, Miller RS, Mende K, *et al.* Antimicrobial susceptibilities of geographically diverse clinical human isolates of *Leptospira*. Antimicrob Agents Chemother 2008;52:2750-4.
17. Suputtamongkol Y, Niwattayakul K, Suttinont C, Losuwanaluk K, Limpaiboon R, Chierakul W, *et al.* An open, randomized, controlled trial of penicillin, doxycycline, and cefotaxime for patients with severe leptospirosis. Clin Infect Dis 2004;39:1417-24.
18. Sejvar J, Bancroft E, Winthrop K, Bettinger J, Bajani M, Bragg S, *et al.* Leptospirosis in "Eco-Challenge" athletes, Malaysian Borneo, 2000. Emerg Infect Dis 2003;9:702-7.