

SUPPLEMENTAL TEXT 1

Multiplex PCR assay to screen bacterial colonies on LVW agar.

An assay was used to screen colonies of *Leptospira* spp. growing on LVW agar and categorize these into pathogenic, intermediate or non-pathogenic *Leptospira* species. This was the first of a two-stage process in which colonies positive for pathogenic or intermediate *Leptospira* species were further evaluated using an *rrs* PCR assay and amplicon sequencing as described in the main text. Single colonies of *Leptospira* growing on LVW agar were inoculated into a 3 mL EMJH broth and incubated at 30°C in air for up to 7 days to achieve a *Leptospira* concentration of approximately 10⁸ CFU/mL. DNA was prepared using a boiling method, as follows. The culture broth was centrifuged at 16,100 × *g* for 5 minutes, the supernatant discarded and the deposit suspended in 50 µL of sterile water. This was boiled at 100°C for 5 minutes using a T100™ Thermal Cycler (BIO-RAD, USA), centrifuged at 16,100 × *g* for 5 minutes, and the supernatant containing DNA transferred into a new 1.5 mL tube.

The multiplex PCR assay was based on *rrs* and *lipL32* genes and used six primers described previously.¹⁻³ These included four *rrs* primers (a single forward [rrs-ini] and three reverse primers [rrs-end, rrs-inner-R and rrs-outer-R]),^{1,2} and two *lipL32* primers (LipL32-45F and LipL32-286R).³ The single forward and outermost reverse *rrs* primers (rrs-end) anneal to all *Leptospira* species to give a 1483bp *rrs* amplicons.² The two additional reverse *rrs* primers (rrs-inner-R and rrs-outer-R) anneal to pathogenic and intermediate *Leptospira* species but not to non-pathogens to give an additional two amplicons (824bp and 573bp).¹ LipL32 is specific to pathogenic *Leptospira* species as *lipL32* primers designed by Stoddard and colleagues could not amplify the gene encoding LipL32 homolog of the intermediate *Leptospira*.^{3,4} The *lipL32* primers give a single 242bp amplicon from pathogens alone.³ This gives the following pattern of bands: pathogenic species, 4 bands; intermediate species, 3 bands; non-pathogenic species, 1 band.

The multiplex PCR assay was performed as follows. A 15 µL PCR reaction contained 200 µM of dNTP, 1.5 unit of Tag DNA polymerase (Roche, USA), and 1 pmol of each primer with the exception of rrs-outer-R (0.5 pmol) and rrs-inner-R (2 pmol). Cycling conditions were as follows: one cycle of 95°C for 2 minutes, 30 cycles of 95°C for 10 seconds, 50°C for 15 seconds and 72°C for 45 seconds, and one cycle of 72°C for 7 minutes. PCR products were visualised by 2% agarose gel electrophoresis.

Analytical specificity of the multiplex PCR assay was determined using DNA extracted from *Leptospiraceae* including 11 *Leptospira* species (seven pathogens: *L. interrogans* serovar Lai strain Lai, *L. kirschneri* serovar Cynopteri strain 3522 C, *L.*

borgpetersenii serovar Javanica strain Veldrat Batavia 46, *L. santarosai* serovar Shermani strain 1342K, *L. weilii* serovar Sarmin strain Sarmin, *L. noguchii* serovar Fortbragg strain Fort Bragg and *L. alstonii* serovar Pingchang strain 80-412; two intermediate group: *L. inadai* serovar Lyme strain 10 and *L. wolffii* strain H2; two non-pathogens: *L. biflexa* serovar Patoc strain Patoc I and *L. wobalchii* serovar Codice strain CDC), *Turneriella parva* serovar Parva strain H, and pathogenic organisms that are common causes of septicemia in our setting area (*Orientia tsutsugamushi*, *Staphylococcus aureus* and *Escherichia coli*). The pathogenic and intermediate group *Leptospira* species gave the predicted amplification patterns (4 bands and 3 bands, respectively). The 2 non-pathogenic *Leptospira* species and *Turneriella parva* gave a single 1483bp amplicon. No PCR product was amplified from *O. tsutsugamushi*, *S. aureus* or *E. coli*. This confirmed that the assay could be used to differentiate pathogenic and intermediate *Leptospira* species from non-pathogenic *Leptospira* species and *Turneriella parva*.

The multiplex PCR assay was performed on 140 single colonies, as described in the main text. Positive and negative controls were included in each run. The positive control used was genomic DNA of *L. interrogans* serovar Lai strain Lai. The results of multiplex screening were supported by *Leptospira* species classification based on *rrs* sequence. All 6 samples belonging to intermediate *Leptospira* species (including samples closely related to *L. licerasiae*) gave a 3-band pattern, and a single sample belonging to a novel putative pathogenic *Leptospira* species gave a 4-band pattern.

REFERENCES

1. Boonsilp S, Thaipadungpanit J, Amornchai P, Wuthiekanun V, Chierakul W, Limmathurotsakul D, Day NP, Peacock SJ, 2011. Molecular detection and speciation of pathogenic *Leptospira* spp. in blood from patients with culture-negative leptospirosis. *BMC Infect Dis* 11: 338.
2. Thaipadungpanit J, Wuthiekanun V, Chierakul W, Smythe LD, Petkanchanapong W, Limpaboon R, Apiwatanaporn A, Slack AT, Suputtamongkol Y, White NJ, Feil EJ, Day NP, Peacock SJ, 2007. A dominant clone of *Leptospira interrogans* associated with an outbreak of human leptospirosis in Thailand. *PLoS Negl Trop Dis* 1: e56.
3. Stoddard RA, Gee JE, Wilkins PP, McCaustland K, Hoffmaster AR, 2009. Detection of pathogenic *Leptospira* spp. through TaqMan polymerase chain reaction targeting the LipL32 gene. *Diagn Microbiol Infect Dis* 64: 247–255.
4. Matthias MA, Ricardi JN, Cespedes M, Diaz MM, Galloway RL, Saito M, Steigerwalt AG, Patra KP, Ore CV, Gotuzzo E, Gilman RH, Levett PN, Vinetz JM, 2008. Human leptospirosis caused by a new, antigenically unique *Leptospira* associated with a *Rattus* species reservoir in the Peruvian Amazon. *PLoS Negl Trop Dis* 2: e213.

SUPPLEMENTAL TABLE 1
rrs sequences from GenBank data used in phylogenetic analysis to identify *Leptospira* species

Group	Species	GenBank ID
Pathogenic <i>Leptospira</i>	<i>L. alexanderi</i>	DQ991482
	<i>L. alstoni</i>	AY631881
	<i>L. borgpetersenii</i>	AM050573
	<i>L. interrogans</i>	AE010300, FJ154564
	<i>L. kirschneri</i>	FJ154572, AY461878
	<i>L. noguchii</i>	FJ154588, FJ154582, EU349495
	<i>L. santarosai</i>	AY996805, AY631883, AY461888, Z21649, FJ154576
Putative pathogenic <i>Leptospira</i>	<i>L. weilii</i>	FJ154590, DQ483058
	<i>L. kmetyi</i>	AB279549
Intermediate <i>Leptospira</i>	<i>L. broomii</i>	AY792329
	<i>L. fainei</i>	AY631885, U60594
	<i>L. inadai</i>	AY631891
	<i>L. licerasiae</i>	EF612280
	<i>L. wolffii</i>	EF025496
	<i>L. biflexa</i>	CP000786, FJ154577
	<i>L. meyeri</i>	AY631892
Non-pathogenic <i>Leptospira</i> and other species of <i>Leptospiraceae</i>	<i>L. terpstrae</i>	AY631888
	<i>L. vanthielii</i>	AY631897
	<i>L. wolbachii</i>	AY631890
	<i>L. yanagawae</i>	AY631882
	<i>Leptonema illini</i>	NR043139
	<i>Turneriella parva</i>	AY293856
	Unculturable bacteria	FJ164045, DQ065390, FJ820401

SUPPLEMENTAL TABLE 2
rrs sequences of *Leptospira* species submitted to GenBank

Group	Sample code-isolate code	Species	GenBank ID
Unculturable <i>Leptospira</i>	65 nested PCR-positive samples	Unknown	JX857331-JX857395
Pathogenic <i>Leptospira</i>	NP-29-Q1	Unknown	JX857402
	NP-29-Q3	Unknown	JX857403
Intermediate <i>Leptospira</i>	NP-46-Q127	<i>L. licerasiae</i>	JX857396
	NP-49-Q74	<i>L. wolffii</i>	JX857397
	NP-63-Q40	Probable <i>L. wolffii</i>	JX857398
	NP-64-Q41	Unknown	JX857399
	NP-21-Q103	<i>L. licerasiae</i>	JX857400
	NP-30-Q29	Unknown	JX857401
	NP-30-Q61	Unknown	JX857408

SUPPLEMENTAL TABLE 3

Polymorphic sites and nucleotide distance between 6 *Leptospira rrs* sequences generated in this study versus reference sequences for *L. wolffii* and *L. licerasiae*

	Polymorphic sites*																		Nucleotide distance† (%)		
																			<i>L. wolffii</i>	<i>L. licerasiae</i>	
	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	2	3				
	3	3	8	9	9	9	0	2	2	2	5	6	7	7	8	8	8	8			
	4	7	3	4	5	7	8	4	6	7	3	1	1	2	1	2	2	7	1	<i>L. wolffii</i>	<i>L. licerasiae</i>
<i>L. wolffii</i>	C	A	T	A	C	G	A	T	C	G	A	A	T	C	G	A	G	C	A		
<i>L. licerasiae</i>	C	G	.	0.45	
NP-21	C	G	.	0.45	0
NP-46	C	G	.	0.45	0
NP-49	0	0.45
NP-63	C/T	A/G	0	0.45
NP-64	T	G	C	T	.	A	G	C	T	A	T	G	C	T	T	G	C	G	.	3.84	3.39
NP-30	T	.	.	.	T	.	C	G	.	0.90	0.45

*A total of 19 polymorphic sites were demonstrated based on the 443-nucleotide fragment of the *rrs* gene. The vertical numbers indicate the nucleotide positions, and a dot indicates an identical nucleotide at that position.

†Pairwise comparisons of nucleotide distance between 6 floodwater isolates (belonging to intermediate group *Leptospira*) and 2 reference sequences (*L. wolffii* [EF025496] and *L. licerasiae* [EF612280]) were analysed base on the 443-nucleotide fragment of the *rrs* gene. NP-30 and NP-64 were most closely related to *L. licerasiae* with a nucleotide distance of 0.45% and 3.39%, respectively.

SUPPLEMENTAL TABLE 4
Results of direct PCR assay and *Leptospira* culture for 110 water samples

Sample ID	Direct PCR assay	<i>Leptospira</i> spp. identified from direct PCR amplicons	<i>Leptospira</i> culture	<i>Leptospira</i> group identified by multiplex PCR screening	Species identification for pathogenic and intermediate <i>Leptospira</i>
				(number of colonies tested)	
NP01	negative		negative		
NP02	positive	unculturable <i>Leptospira</i>	negative		
NP04	positive	unculturable <i>Leptospira</i>	positive	non-pathogen (4)	
NP05	negative		positive	non-pathogen (2)	
NP06	positive	unculturable <i>Leptospira</i>	negative		
NP09	negative		positive	non-pathogen (1)	
NP10	positive	unculturable <i>Leptospira</i>	negative		
NP12	negative		positive	non-pathogen (2)	
NP18	negative		negative		
NP20	positive	unculturable <i>Leptospira</i>	positive	non-pathogen (2)	
NP21	positive	unsuccessful in DNA sequencing	positive	intermediate (1)	<i>L. licerasiae</i>
NP22	negative		positive	non-pathogen (1)	
NP23	positive	unculturable <i>Leptospira</i>	negative		
NP24	positive	unculturable <i>Leptospira</i>	positive	non-pathogen (3)	
NP25	positive	unculturable <i>Leptospira</i>	positive	non-pathogen (1)	
NP26	positive	unculturable <i>Leptospira</i>	positive	non- <i>Leptospiraceae</i> spirochetes (2), non-pathogen (1)	
NP27	positive	unculturable <i>Leptospira</i>	positive	non-pathogen (2)	
NP28	positive	unculturable <i>Leptospira</i>	positive	non-pathogen (2)	
NP29	positive	unculturable <i>Leptospira</i>	positive	pathogen (2)	closely related to <i>L. alstonii</i>
NP30	positive	unculturable <i>Leptospira</i>	positive	non-pathogen (1), intermediate (2)	closely related to <i>L. licerasiae</i>
NP31	positive	unculturable <i>Leptospira</i>	positive	non-pathogen (1)	
NP32	negative		positive	non-pathogen (2)	
NP33	positive	unculturable <i>Leptospira</i>	negative		
NP34	negative		positive	non-pathogen (1)	
NP35	negative		positive	non- <i>Leptospiraceae</i> spirochetes (2)	
NP36	negative		positive	non-pathogen (2)	
NP37	negative		positive	non-pathogen (1)	
NP38	positive	unculturable <i>Leptospira</i>	positive	non-pathogen (1)	
NP39	negative		positive	non-pathogen (1)	
NP40	negative		positive	non-pathogen (2)	
NP41	negative		positive	non-pathogen (2)	
NP42	positive	unculturable <i>Leptospira</i>	positive	non-pathogen (2)	
NP43	negative		positive	non-pathogen (1)	
NP44	positive	unsuccessful in DNA sequencing	positive	non-pathogen (2)	
NP45	negative		positive	non-pathogen (1)	
NP46	positive	unsuccessful in DNA sequencing	positive	intermediate (1)	<i>L. licerasiae</i>
NP47	positive	unculturable <i>Leptospira</i>	positive	non-pathogen (2)	
NP48	negative		negative		
NP49	positive	unsuccessful in DNA sequencing	positive	non-pathogen (1), intermediate (1)	<i>L. wolffii</i>
NP50	negative		positive	non-pathogen (1)	
NP51	negative		positive	non-pathogen (1)	
NP52	positive	unsuccessful in DNA sequencing	positive	non-pathogen (1)	
NP53	positive	unculturable <i>Leptospira</i>	positive	non-pathogen (1)	
NP54	positive	unculturable <i>Leptospira</i>	positive	non-pathogen (1)	
NP55	positive	unculturable <i>Leptospira</i>	positive	non-pathogen (1)	
NP56	positive	unculturable <i>Leptospira</i>	positive	non-pathogen (1)	
NP57	positive	unculturable <i>Leptospira</i>	positive	non- <i>Leptospiraceae</i> spirochetes (1), non-pathogen (1)	
NP58	negative		positive	non-pathogen (1)	
NP59	negative		negative		
NP60	negative		positive	non-pathogen (2)	
NP61	negative		positive	non-pathogen (1)	
NP62	negative		negative		
NP63	negative		positive	intermediate (1)	<i>L. wolffii</i>
NP64	negative		positive	intermediate (1)	closely related to <i>L. licerasiae</i>
NP65	negative		positive	non- <i>Leptospiraceae</i> spirochetes (1)	
NP66	negative		positive	non-pathogen (2)	
NP67	negative		positive	non-pathogen (2)	
NP68	negative		positive	non-pathogen (1)	
NP69	positive	unculturable <i>Leptospira</i>	negative		
NP70	positive	unculturable <i>Leptospira</i>	positive	non-pathogen (1)	
NP71	positive	unsuccessful in DNA sequencing	negative		
NP72	positive	unsuccessful in DNA sequencing	positive	non-pathogen (1)	

(continued)

SUPPLEMENTAL TABLE 4
Continued

Sample ID	Direct PCR assay	<i>Leptospira</i> spp. identified from direct PCR amplicons	<i>Leptospira</i> culture	<i>Leptospira</i> group identified by multiplex PCR screening	Species identification for pathogenic and intermediate <i>Leptospira</i>
				(number of colonies tested)	
NP73	positive	unculturable <i>Leptospira</i>	positive	non-pathogen (1)	
NP74	positive	unculturable <i>Leptospira</i>	positive	non-pathogen (1)	
NP75	positive	unculturable <i>Leptospira</i>	negative		
NP76	positive	unculturable <i>Leptospira</i>	positive	non-pathogen (1)	
NP77	positive	unculturable <i>Leptospira</i>	positive	non-pathogen (1)	
NP78	positive	unculturable <i>Leptospira</i>	positive	non-pathogen (1)	
NP79	positive	unsuccessful in DNA sequencing	positive	non-pathogen (2)	
NP80	positive	unsuccessful in DNA sequencing	positive	non-pathogen (2)	
DM01	positive	unculturable <i>Leptospira</i>	positive	non-pathogen (1)	
DM02	positive	unculturable <i>Leptospira</i>	positive	non-pathogen (1)	
DM03	positive	unculturable <i>Leptospira</i>	negative		
DM04	positive	unculturable <i>Leptospira</i>	positive	non-pathogen (2)	
DM05	negative		negative		
DM06	positive	unculturable <i>Leptospira</i>	negative		
DM07	positive	unculturable <i>Leptospira</i>	positive	non- <i>Leptospiraceae</i> spirochetes (2)	
DM08	positive	unculturable <i>Leptospira</i>	positive	non-pathogen (2)	
DM09	positive	unculturable <i>Leptospira</i>	negative		
DM10	positive	unculturable <i>Leptospira</i>	positive	non-pathogen (1)	
DM11	negative		negative		
DM12	positive	unculturable <i>Leptospira</i>	positive	non-pathogen (2)	
DM13	positive	unculturable <i>Leptospira</i>	negative		
DM14	positive	unculturable <i>Leptospira</i>	negative		
DM15	positive	unculturable <i>Leptospira</i>	positive	non-pathogen (1)	
DM16	positive	unculturable <i>Leptospira</i>	negative		
DM17	negative		positive	non-pathogen (2)	
DM18	positive	unculturable <i>Leptospira</i>	positive	non-pathogen (2)	
DM19	positive	unculturable <i>Leptospira</i>	positive	non-pathogen (1)	
DM20	positive	unculturable <i>Leptospira</i>	positive	non-pathogen (1)	
DM21	positive	unculturable <i>Leptospira</i>	positive	non- <i>Leptospiraceae</i> spirochetes (3)	
DM22	positive	unculturable <i>Leptospira</i>	positive	non-pathogen (1)	
DM23	positive	unculturable <i>Leptospira</i>	positive	non-pathogen (3)	
DM24	positive	unculturable <i>Leptospira</i>	positive	non-pathogen (1)	
DM25	positive	unsuccessful in DNA sequencing	positive	non-pathogen (1)	
DM26	positive	unculturable <i>Leptospira</i>	positive	non-pathogen (1)	
DM27	positive	unculturable <i>Leptospira</i>	positive	non-pathogen (1)	
DM28	positive	unculturable <i>Leptospira</i>	positive	non- <i>Leptospiraceae</i> spirochetes (3)	
DM29	positive	unculturable <i>Leptospira</i>	positive	non- <i>Leptospiraceae</i> spirochetes (2), non-pathogen (3)	
DM30	positive	unculturable <i>Leptospira</i>	positive	non- <i>Leptospiraceae</i> spirochetes (4)	
DM31	positive	unculturable <i>Leptospira</i>	positive	non-pathogen (1)	
DM32	positive	unculturable <i>Leptospira</i>	positive	non-pathogen (1)	
DM33	positive	unculturable <i>Leptospira</i>	positive	non-pathogen (1)	
DM34	positive	unculturable <i>Leptospira</i>	positive	non-pathogen (2)	
DM35	negative		negative		
DM36	positive	unculturable <i>Leptospira</i>	positive	non-pathogen (1)	
DM37	positive	unculturable <i>Leptospira</i>	positive	non-pathogen (1)	
DM38	positive	unsuccessful in DNA sequencing	positive	non-pathogen (2)	
DM39	positive	unculturable <i>Leptospira</i>	positive	non-pathogen (3)	
DM40	positive	unculturable <i>Leptospira</i>	negative		