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 $16,100 \times g$ 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 T100TM Thermal Cycler (BIO-RAD, USA), centrifuged at $16,100 \times g$ 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 colleages 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 μ L PCR reaction contained 200 μ M 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 nonpathogenic 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

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- 4. Matthias MA, Ricaldi 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.

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

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

SUPPLEMENTAL TABLE 2 rrs sequences of Leptospira species submitted to GenBank

Group	Sample code-isolate code	Species	GenBank ID
Unculturable Leptospira	65 nested PCR-positive samples	Unknown	JX857331-JX857395
Pathogenic Leptospira	NP-29-Q1	Unknown	JX857402
	NP-29-Q3	Unknown	JX857403
Intermediate Leptospira	NP-46-Q127	L. licerasiae	JX857396
	NP-49-Q74	L. wolffii	JX857397
	NP-63-Q40	Probable L. wolffii	JX857398
	NP-64-Q41	Unknown	JX857399
	NP-21-Q103	L. licerasiae	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† (%)										
							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	8		
	4	7	3	4	5	7	8	4	6	7	3	1	1	2	1	2	2	7	1	L. wolffii	L. licerasiae
L. wolffii	С	А	Т	А	С	G	А	Т	С	G	А	А	Т	С	G	А	G	С	А		
L. licerasiae																	С	G		0.45	
NP-21																	С	G		0.45	0
NP-46																	С	G		0.45	0
NP-49																				0	0.45
NP-63					C/T														A/G	0	0.45
NP-64	Т	G	С	Т		А	G	С	Т	А	Т	G	С	Т	Т	G	С	G		3.84	3.39
NP-30											Т				Т		С	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.

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	NP70		unculturable Leptospira	positive	non-pathogen (1)	
NP72 positive unsuccessful in DNA sequencing positive non pathogen (1)		1				
14.72 positive unsuccessiu in DAA sequencing positive non-pathogen (1)	NP72	positive	unsuccessful in DNA sequencing	positive	non-pathogen (1)	

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

SUPPLEMENTAL TABLE 4
Continued

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