Parasitology

cambridge.org/par

Research Article

*These first authors contributed equally to this article.

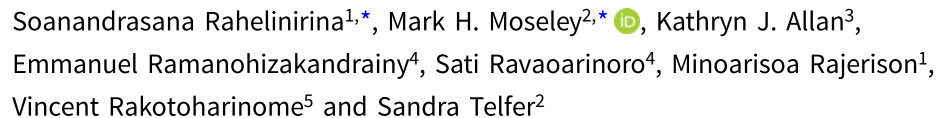
Cite this article: Rahelinirina S, Moseley MH, Allan KJ, Ramanohizakandrainy E, Ravaoarinoro S, Rajerison M, Rakotoharinome V, Telfer S (2019). *Leptospira* in livestock in Madagascar: uncultured strains, mixed infections and small mammal-livestock transmission highlight challenges in controlling and diagnosing leptospirosis in the developing world. *Parasitology* **146**, 1707–1713. https://doi.org/10.1017/S0031182019001252

Received: 2 May 2019
Revised: 22 July 2019
Accepted: 20 August 2019
First published online: 26 September 2019

Key words:
Africa; Madagascar; pathogen; spillover; spirochaete; Tanzania; veterinary; zoonosis

Author for correspondence: Mark H. Moseley, E-mail: mark.moseley@abdn.ac.uk

Leptospira in livestock in Madagascar: uncultured strains, mixed infections and small mammal-livestock transmission highlight challenges in controlling and diagnosing leptospirosis in the developing world



Soanandrasana Rahelinirina[1,*], Mark H. Moseley[2,*], Kathryn J. Allan[3], Emmanuel Ramanohizakandrainy[4], Sati Ravaoarinoro[4], Minoarisoa Rajerison[1], Vincent Rakotoharinome[5] and Sandra Telfer[2]

[1]Plague Unit, Institut Pasteur de Madagascar, 101 Antananarivo, Madagascar; [2]School of Biological Sciences, University of Aberdeen, Aberdeen, AB24 2TZ, UK; [3]Institute of Biodiversity, Animal Health and Comparative Medicine, University of Glasgow, University Avenue, Glasgow, G12 8QQ, UK; [4]University of Antananarivo, BP 566, Ankatso, 101 Antananarivo, Madagascar and [5]Ministere de l'Agriculture et de l'Elevage, BP 1453, Analakely, Antananarivo, Madagascar

Abstract

In developing countries, estimates of the prevalence and diversity of *Leptospira* infections in livestock, an important but neglected zoonotic pathogen and cause of livestock productivity loss, are lacking. In Madagascar, abattoir sampling of cattle and pigs demonstrated a prevalence of infection of 20% in cattle and 5% in pigs by real-time PCR. In cattle, amplification and sequencing of the *Leptospira*-specific *lfb1* gene revealed novel genotypes, mixed infections of two or more *Leptospira* species and evidence for potential transmission between small mammals and cattle. Sequencing of the *secY* gene demonstrated genetic similarities between *Leptospira* detected in Madagascar and, as yet, uncultured *Leptospira* strains identified in Tanzania, Reunion and Brazil. Detection of *Leptospira* DNA in the same animal was more likely in urine samples or pooled samples from four kidney lobes relative to samples collected from a single kidney lobe, suggesting an effect of sampling method on detection. In pigs, no molecular typing of positive samples was possible. Further research into the epidemiology of livestock leptospirosis in developing countries is needed to inform efforts to reduce human infections and to improve livestock productivity.

Introduction

Zoonoses account for 61% of infectious diseases of humans and 75% of emerging infectious diseases (Taylor *et al.*, 2001; Woolhouse and Gowtage-Sequeria, 2005). Endemic zoonoses, characterised by a widespread distribution and frequent transmission between animals and humans, include some of the most important diseases of poverty (Maudlin *et al.*, 2009; ILRI, 2012). However, in the poorest communities in the developing world, a lack of surveillance and control (Halliday *et al.*, 2012) and misdiagnosis (Maudlin *et al.*, 2009) result in these diseases being amongst the most neglected in the world (ILRI, 2012). It has been suggested that, in terms of human health impacts, livestock productivity loss and amenity to agricultural intervention, leptospirosis, a neglected endemic zoonosis causing an estimated 1 million cases of human disease annually (Costa *et al.*, 2015), is the second most important zoonosis globally (ILRI, 2012). Although rodents are frequently implicated as the key reservoir hosts of *Leptospira* (Picardeau, 2017), recent evidence suggests that, in rural Africa, livestock may also be significant reservoir hosts (Allan *et al.*, 2018).

Leptospira are phylogenetically delineated into 22 species, 10 of which are pathogenic, and further divided into more than 300 serovars which may demonstrate specific, but not absolute, host preferences (Picardeau, 2017). In Madagascar, four *Leptospira* species have been identified in terrestrial small mammals (Rahelinirina *et al.*, 2010; Dietrich *et al.*, 2014; Moseley *et al.*, 2018) and a molecular link demonstrated between a recent acute, severe case of human leptospirosis (Pagès *et al.*, 2015) and small mammals (Moseley *et al.*, 2018). However, a recent household cross-sectional serosurvey in Madagascar identified contact with cattle as the only significant risk factor [OR = 3, 95% CI (1.03–10.03)] for human exposure despite serological typing based on Microscopic Agglutination Testing (MAT) identifying Icterohaemorrhagiae, a serogroup traditionally associated with rodents, as the predominant serogroup (Ratsitorahina *et al.*, 2015).

Therefore, understanding the role of livestock in the epidemiology of leptospirosis is critical to understanding public health risks and informing control strategies. Vaccination of livestock is used to both improve productivity and to reduce zoonotic risk from livestock (Marshall and Chereshsky, 1996). However, recent studies have demonstrated that commercial leptospirosis

© Cambridge University Press 2019

CAMBRIDGE UNIVERSITY PRESS

vaccines may lack efficacy when challenged by autochthonous livestock strains in Brazil (Sonada *et al.*, 2018) and will not reduce human infections from serovars associated with wildlife (Thornley *et al.*, 2002). Therefore, to inform control measures, we used abattoir sampling and molecular methods to determine the prevalence and diversity of *Leptospira* in livestock in Madagascar and their phylogenetic relationship to *Leptospira* identified in livestock from similar studies in other developing countries.

Materials and methods

Abattoir sampling

In 2015, we estimated *Leptospira* prevalence by sampling a total of 205 livestock: 25 cattle and 25 pigs sampled at each of three abattoirs around the capital, Antananarivo, and 30 cattle and 25 pigs sampled at the abattoir in the town of Moramanga. Livestock presented at abattoirs were sourced from a wide geographic area (Supplementary Fig. S1). Whole kidneys and urine aspirates were collected and cooled for transportation to a laboratory.

Sample preparation

As *Leptospira* may not be homogeneously distributed within kidney tissue and urinary shedding is intermittent, to advise future studies/surveillance we compared results from three sample preparations: a single kidney excision, a pool of four kidney excisions from different lobes (cattle) or anatomical locations (pigs) and urine. Kidney samples were stored in 95% ethanol and urine samples at −80 °C.

(i). Kidney samples

Extraction of the kidney samples was performed on 40 mg of tissue using the DNeasy Blood and Tissue Kit (Qiagen) as per the manufacturer's instructions with the volumes of buffers ATL and AL increased to account for the increased amount of tissue extracted and the volume of elution buffer reduced to 100 μL to concentrate the DNA.

For preparations from a single lobe/location, 40 mg of tissue from the corticomedullary junction was extracted. For pooled samples of four kidney lobes, 40 mg of tissue from each lobe was placed in a sterile 1.5 mL Eppendorf tube containing 400 μL of the ATL lysis buffer and ground with a glass pestle to obtain a homogenate. One hundred microliters of the homogenate were then placed in a new sterile 1.5 mL Eppendorf and extracted as outlined above. To monitor inhibition, 1.5 μL of DNA extraction control 560 (Bioline) was added to each extraction.

(ii). Urine samples

After slaughter urine samples were obtained by aspiration of 2 mL of urine from the bladder using sterile needles and 2 mL syringes. If samples arrived at the laboratory within 24 hours they were transferred to a clean cryotube and stored at −80 °C. For the Moramanga abattoir, this was not possible. Consequently, samples were centrifuged at 7000 rpm for 20 min before discarding the supernatant and adding 200 μL of TE buffer (Sigma-Aldrich). The samples were then kept at +4 °C, before transferring to the laboratory in a cool box with ice packs.

Extraction of the urine samples was performed using the same protocol as for the kidney samples with the exception of the following pre-extraction step. Samples frozen at −80 °C were incubated at 67 °C for one hour before 1 mL was transferred to a 2 mL Eppendorf. Samples were then centrifuged at 7000 rpm for 10 min. After discarding the supernatant, the pellet was suspended in 200 μL of TE buffer. The re-suspended pellet was centrifuged again at 7000 rpm for 10 min and the supernatant discarded. The pellet was then re-suspended again with 100 μL of TE buffer, 50 μL of lysozyme (10 μg mL^{-1}), 50 μL of mutanolysin (4 KU mL^{-1}), 4 μL of lysostaphin and incubated at 37 °C for one hour. After addition of 20 μL of proteinase K and 180 μL of AL buffer (Qiagen) and vortexing, samples were incubated at 56 °C for 10 min. The final step involved the addition of 200 μL of 95% ethanol and vortexing before following the extraction protocol used for kidney samples.

Leptospira detection and DNA sequencing

Prevalence estimates were obtained using a 16s (*rrs*) qPCR (Smythe *et al.*, 2002) as described previously (Moseley *et al.*, 2018). Each sample preparation (single lobe/location, four lobes/location and urine sample) was tested separately. An individual animal was identified as infected if any of the sample preparations tested positive. χ^2 tests were used to test for differences in prevalence estimates between cattle and pigs and between prevalence estimates from different sample preparations within the same host. Mann–Whitney–Wilcoxon tests were used to determine whether parasite load, as measured by 16s qPCR amplification threshold (Ct), affected typing success. Statistical analyses were performed using R version 3.4.1 software (R Core Team).

Amplification of 200–300 bp of the *lfb1* gene (Merien *et al.*, 2005; Moseley *et al.*, 2018) was then performed on positive kidney and urine sample from each animal and on positive livestock samples previously identified in Tanzania (Allan *et al.*, 2018). Initially, samples were tested using *lfb*1 primers (Merien *et al.*, 2005) designed to detect all pathogenic *Leptospira*. Previous molecular studies in Africa have identified *L. borgpetersenii*, *L. kirschneri* and a *L. kirschneri*-like species in livestock (Allan *et al.*, 2018) and suggest that existing *lfb1* primers fail to amplify *L. borgpetersenii* in some cases (Moseley *et al.*, 2018). Therefore, redesigned *lfb1* forward primers targeting *L. kirschneri* and *L. borgpetersenii* (Moseley *et al.*, 2018) were subsequently used on samples that tested negative using the standard *lfb1* qPCR. In addition, to test for mixed infections, samples infected with *L. borgpetersenii* were tested using *L. kirschneri* targeting primers and vice versa. All *lfb1* assays were performed using reaction conditions as previously described (Moseley *et al.*, 2018) and all amplicons were sequenced.

To facilitate comparisons with other studies, amplification and sequencing of the *secY* gene, a widely used target for phylogenetic analysis of *Leptospira* (Victoria *et al.*, 2008), was undertaken. Initial amplification of a ∼450 bp fragment was performed using reaction conditions previously described (Allan *et al.*, 2018). Where initial amplification failed to amplify sufficient material for sequencing, a second round of amplification of a ∼350 bp fragment was performed in a 25 μL reaction volume using a nested forward primer [5′-AATCCATTYTCYCARATYTGGTA-3′] and the first round reverse primer at concentrations of 0.5 μM, 12.5 μL of MyTaq Red mix 2x (Bioline Reagents Ltd), 9.5 μL of molecular grade water and 1 μL of first round product. The thermal profile comprised initial denaturation at 95 °C for 3 min, followed by 20 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s and extension at 72 °C for 1 min, with a final extension at 72 °C for 7 min.

DNA sequence analysis

Phylogenetic analysis of *lfb1* sequences was supplemented with *lfb1* sequences obtained from small mammals in Madagascar (Moseley *et al.*, 2018), a goat opportunistically sampled during

Table 1. Prevalence of *Leptospira* infection using different sample preparations from cattle and pigs

Host	Sample preparations			Overall prevalence (*P*/*n*, 95% CI)
	Sample type	Inhibitors	Prevalence (*P*/*n*, 95% CI)	
Cattle (*n* = 105)	Kidney (1 lobe) (*n* = 105)	11	6% (6/94, 3–13%)	19% (20/105, 13–28%)
	Kidney (4 lobes) (*n* = 105)	3	12% (12/102, 7–20%)	
	Urine (*n* = 99)	3	11% (11/96, 6–20%)	
Pigs (*n* = 100)	Kidney (1 location) (*n* = 100)	1	1% (1/99, 0–6%)	5% (5/100, 2–11%)
	Kidney (4 locations) (*n* = 100)	7	0% (0/93, 0–4%)	
	Urine (*n* = 95)	0	4% (4/95, 2–11%)	

Cattle kidneys were sampled at four lobes and pig kidneys were sampled at four locations (pig kidneys are not lobed). Infection prevalence and 95% confidence intervals (logit method) were determined using the binom package (https://cran.r-project.org/ package = binom). Samples with evidence of inhibition were excluded from prevalence estimate calculations for each sample preparation but each individual animal had at least one sample preparation without evidence of inhibition so every animal was represented in overall prevalence estimates

village-based rodent surveys and four cattle sampled during a pilot study in Antananarivo. In addition, to provide additional *lfb1* sequences from the region and to test for mixed infections, the same *lfb1* assays were used to obtain sequences from 28 livestock sampled in a similar abattoir study in northern Tanzania (Allan *et al*., 2018) and typed using *secY* sequencing. To identify the serovars to which these strains are most closely related, *lfb1* sequences were queried against the NCBI refseq_genomes and nr/nt database using the blastn algorithm and identical or closely related (>99% identity) records with associated serovar information reported.

To place the strains identified in this study in a global context, *secY* sequences were analysed alongside sequences from similar livestock studies from Tanzania (Allan *et al*., 2018), Reunion (Guernier *et al*., 2016) and Brazil (Hamond *et al*., 2015; Guedes *et al*., 2019) (Supplementary Table S1). Reference *secY* sequences (Victoria *et al*., 2008) with 100% identity to any of the *secY* genotypes were included in the phylogenetic analysis. Sequence alignment and phylogenetic analysis were performed using MEGA7 (Kumar *et al*., 2016).

Results

Prevalence estimates and effect of sample preparation

Cattle had a significantly higher overall prevalence than pigs (19 *vs* 5%, $\chi^2 = 8.17$, $P < 0.01$) (Table 1). Cattle had 13 infections detected in kidney samples and 11 in urine samples, with only four individuals testing positive for both sample types. Pooled kidney excisions detected more infections than single excisions (12 *vs* 6%) (Table 1), although the difference was not significant ($\chi^2 = 1.11$, $P = 0.29$), and only one individual tested positive by the single excision but negative by the pooled excision. Of the positive pigs, only one infection was detected in the kidney (single excision), compared to four infections detected in urine.

Typing success

In Madagascar, interpretable *lfb1* and *secY* sequences were obtained from 50% (10/20) and 40% (8/20) of 16s qPCR positive cattle samples respectively. All *secY* sequences were obtained from samples in which an *lfb1* sequence had previously been amplified. In kidney samples, parasite load, as measured by 16s qPCR Ct, had an effect on sequencing success with samples from which sequencing data was obtained having a higher parasite load (median 16s Ct = 33.31, range 32.38–33.99) than samples in which sequencing was unsuccessful (median 16s Ct = 37.85, range 33.95–39.51) ($W = 2$, $P = 0.002$). No such effect was noted in urine samples (median 16s Ct = 35, range 34–37 *vs* median 16s Ct = 37.4, range 33–39) ($W = 13.5$, $P = 0.18$). None of the 16s qPCR positive pig ($n = 5$, 16s Ct range 37–39) samples yielded interpretable *lfb1* or *secY* sequences. Samples from Tanzania identified to be infected with an unknown *L. kirschneri*-like *Leptospira* species based on *secY* sequences (Allan *et al*., 2018), failed to satisfactorily amplify using any of the *lfb1* primer combinations used.

Genetic diversity and mixed infections

Based on *lfb1* sequences, we identified *L. borgpetersenii* and *L. kirschneri* in cattle and *L. interrogans* in a goat sample in Madagascar (Fig. 1). Including *lfb1* sequences from both Madagascan and Tanzanian samples, five *L. borgpetersenii lfb1* clades (A–E) were identified in cattle, two of which (clade A and clade D) were shared between both countries. *L. borgpetersenii* clade A sequences were identical to serovar Hardjo-bovis strain L550, a livestock associated strain (Bulach *et al*., 2006). However, *L. borgpetersenii* clade D had no *lfb1* homologue. Of the remaining three *L. borgpetersenii* clades, two (clade C and clade E) were present in Madagascar, Clade E sequences were identical to *lfb1* sequences previously obtained from small mammals (Moseley *et al*., 2018) and the clade C sequence was identical to a strain previously identified as serovar Tarassovi. The remaining *L. borgpetersenii* clade, detected only in Tanzania, was identical to serovar Hardjo-bovis strain JB197 (Bulach *et al*., 2006).

L. kirschneri lfb1 sequences, obtained from the kidney and urine samples of a single animal from the Moramanga region of Madagascar, demonstrated 99% identity to sequences previously obtained from endemic small mammals (*Hemicentetes semispinosus*) (Moseley *et al*., 2018) in the same region and the *L. interrogans* sequence obtained from a goat was identical to a genotype previously identified predominantly in black rats (*Rattus rattus*) (Moseley *et al*., 2018) and to that obtained from an acute human case of leptospirosis (Pagès *et al*., 2015).

Using the *lfb1* primers targeting different *Leptospira* species, mixed infections were detected in two cattle in Madagascar and one in Tanzania. In Madagascar, *L. kirschneri* and *L. borgpetersenii* clade E were detected in a urine sample from one animal and *L. borgpetersenii* clade A was detected in the urine sample and *L. borgpetersenii* clade E in the kidney sample from another animal. In Tanzania, *L. kirschneri* and *L. borgpetersenii* clade A were detected in a single kidney sample.

Phylogenetic relationship to Leptospira *from other livestock studies in the developing world*

Analysis of *secY* sequences from this study and other studies in northern Tanzania (Allan *et al*., 2018), Reunion island

https://doi.org/10.1017/S0031182019001252 Published online by Cambridge University Press

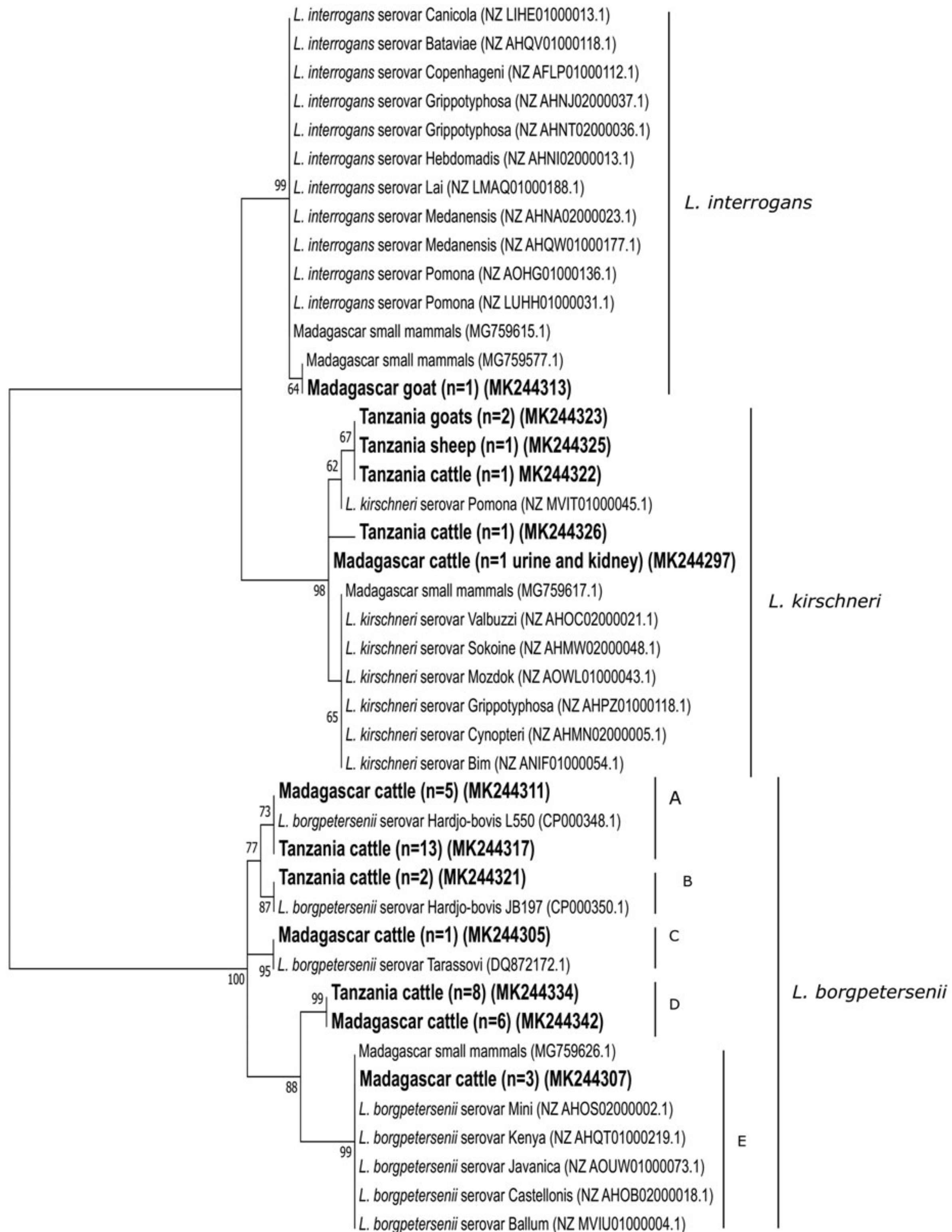


Fig. 1. Maximum likelihood phylogenetic tree of 171 bp *lfb1* sequences (accession numbers MK244296-MK244341) using the Kimura 2-Parameter model with a uniform distribution. Samples from Madagascar and Tanzania are highlighted in bold and the number of animals from which sequences were obtained is indicated. Reference sequences are labelled by *Leptospira* species, serovar and accession number. Nodes are labelled with bootstrap support.

(Guernier *et al.*, 2016) and Brazil (Hamond *et al.*, 2015; Guedes *et al.*, 2019) that used similar molecular approaches identified five recognized pathogenic *Leptospira* species (*L. borgpetersenii, L. santarosai, L. interrogans, L. noguchii, L. kirschneri*) and one *L. kirschneri*-like species infecting cattle (Fig. 2). In Madagascar, four *L. borgpetersenii* secY clades (A–D) were identified. With the exception of clade B, all remaining clades contained *secY* sequences from cattle from other regions of the world. Both clades A and D were detected in cattle from Tanzania (Allan *et al.*, 2018), Reunion (Guernier *et al.*, 2016) and Brazil (Hamond *et al.*, 2015; Guedes *et al.*, 2019). Clade A sequences were identical to *L. borgpetersenii* serovar Hardjo-bovis strains JB197 and L550, strains associated with cattle (Bulach *et al.*, 2006), which *lfb1* sequencing had been able to differentiate in Tanzanian samples (Fig. 1: *L. borgpetersenii* clade A and B). However, clade D sequences matched no reference strains, with sequences in clade

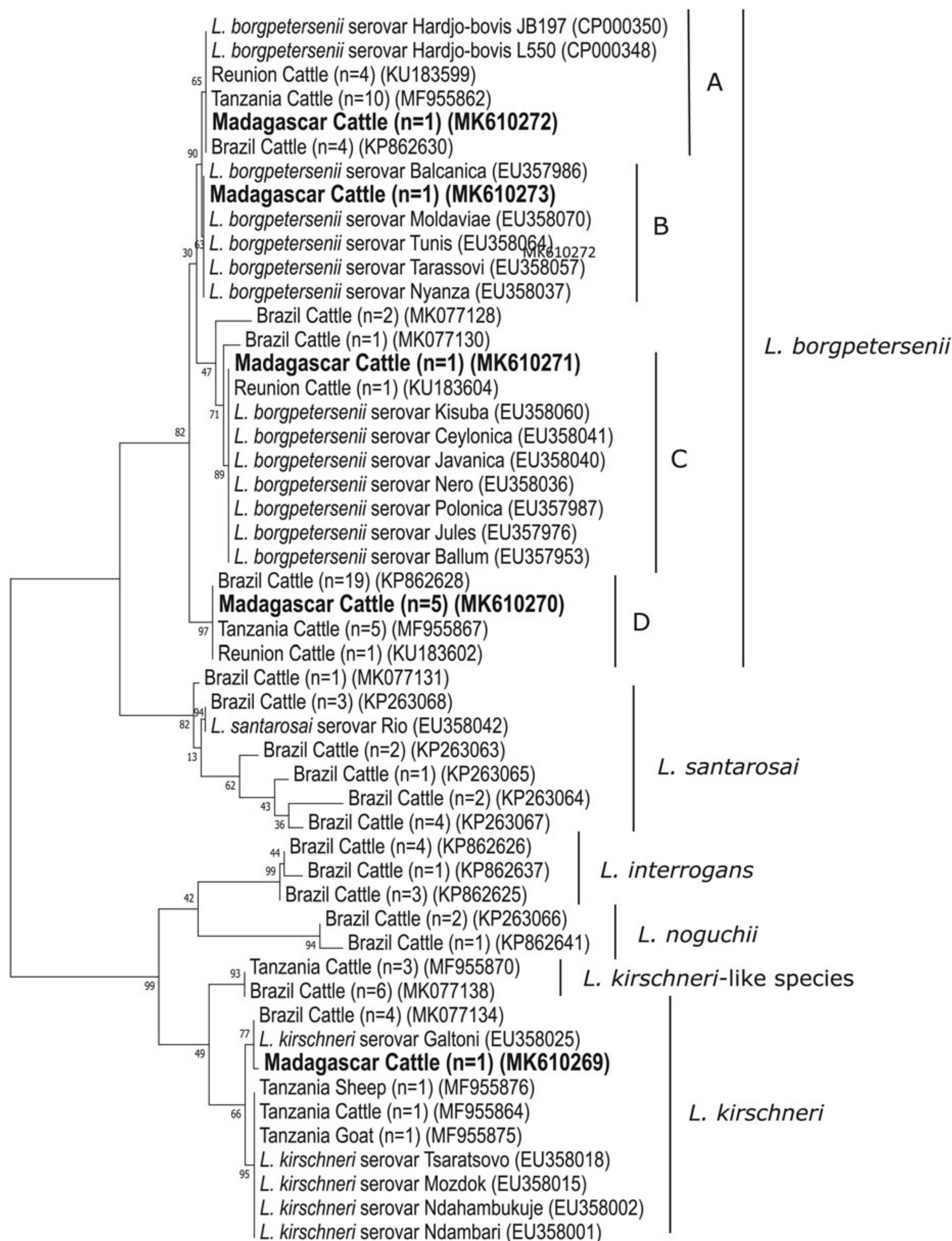


Fig. 2. Maximum likelihood phylogenetic tree of 329 bp *secY* sequences (accession numbers MK244342-MK244344 and MK610269-MKMK610274) using the Tamura 3-Parameter model with a discrete Gamma distribution. Samples from Madagascar are highlighted in bold with the number of animals from which sequences were obtained and a representative accession number indicated. Sequences from similar livestock studies are labelled by country of origin, host, number of sequences and accession number of a representative sequence. Reference sequences (Victoria *et al.*, 2008) are labelled by *Leptospira* species, serovar and accession number. Nodes are labelled with bootstrap support.

B providing the closest match (98% identity). Sequences in clade C were detected in a single animal in this study and a single animal in Reunion (Guernier *et al.*, 2016) and were identical to reference sequences from strains previously associated with small mammals (Victoria *et al.*, 2008). Moreover, the corresponding *lfb1* sequence from Madagascan cattle (Fig. 1, *L. borgpetersenii* clade E) was identical to numerous *lfb1* sequences obtained from Madagascan small mammals (Moseley *et al.*, 2018). Although the single *L. kirschneri* sequence from this study was novel, it was closely related to sequences obtained from cattle in the Brazilian Amazon (Guedes *et al.*, 2019) and to *L. kirschneri* serovar Galtoni, which was obtained from a cattle kidney sample

https://doi.org/10.1017/S0031182019001252 Published online by Cambridge University Press

in Argentina (Victoria *et al.*, 2008), differing by a single synonymous polymorphism. *L. santarosai* and *L. noguchii*, which have been identified in Brazil (Hamond *et al.*, 2015; Guedes *et al.*, 2019) and a *L. kirschneri*-like species recently identified in both Brazil (Guedes *et al.*, 2019) and Tanzania (Allan *et al.*, 2018) were not identified in Madagascan cattle.

Discussion

We show that cattle have a higher prevalence of *Leptospira* infection than pigs in Madagascar and are potential reservoir hosts for a diversity of *Leptospira* with genetic similarities to *Leptospira* strains identified in cattle in other tropical regions of the world, some of which are genetically distinct from reference strains. Increased probability of detection of *Leptospira* infections in sample preparations that included multiple kidney lobes or urine rather than single kidney lobes, suggest a localized distribution for *Leptospira* infections in cattle kidneys or low *Leptospira* loads in single samples. These findings support previous studies (Guedes *et al.*, 2019), where prevalence estimates from urine samples (14.9%, 31/208) were higher than those obtained from kidney samples (5.8%, 12/208), and highlight the importance of considering sample collection methods when evaluating prevalence estimates. *Leptospira* sequencing success was dependent on parasite load and the inability to obtain *Leptospira* sequence data from pigs was likely due to the low parasite load in these samples.

The identification of strains in Madagascar, Tanzania, Reunion and Brazil, such as *L. borgpetersenii* clade D (Fig. 2), genetically distinct from any reference strains, has implications for the control and diagnosis of leptospirosis in the developing world. In Brazil, it has been demonstrated that the efficacy of commercial vaccines is poor in the face of autochthonous strain challenge (Sonada *et al.*, 2018) and it is likely that commercial vaccines are likely to perform as poorly in Madagascar and other developing countries. Moreover, the inclusion of local isolates in antigen panels for serological assays, such as MAT, can dramatically increase the serological detection of infections (Mgode *et al.*, 2015). Therefore, the identification of strains with no associated reference strains suggests that existing antigen panels might underestimate the prevalence of human infections.

In addition to strains associated with livestock, we also identified the infection of cattle with strains previously detected in small mammals. For example, in Madagascar, a *L. interrogans* strain common in small mammals (Moseley *et al.*, 2018) and identified in an acute human case of leptospirosis (Pagès *et al.*, 2015) was also identified in a goat. Moreover, *secY* sequencing identified a *L. borgpetersenii* strain previously detected in small mammals (Victoria *et al.*, 2008) in cattle in this study and in Reunion (Guernier *et al.*, 2016) and *lfb1* sequencing confirmed this strain as identical to *L. borgpetersenii* previously identified in small mammals in Madagascar (Moseley *et al.*, 2018). Thus, our results suggest transmission of at least some *Leptospira* strains between small mammals and livestock, and raise the potential that livestock may play a role in the transmission of these strains. For example, due to their large size, livestock could act as amplification hosts for small mammal associated strains by increasing environmental contamination through urinary shedding after incidental infection or could act as reservoir hosts in their own right, possibly explaining why contact with cattle has been identified as a risk factor for human *Leptospira* infection in Madagascar despite serological typing identifying serogroup Icterohaemorrhagiae, a serogroup associated with rodents, as the predominant serogroup (Ratsitorahina *et al.*, 2015). In contrast, in Tanzania, where small mammals sampled tested negative for *Leptospira* infection (Allan *et al.*, 2018), no evidence was found for infection of livestock with strains previously associated with small mammals.

We also confirm the presence of mixed infections in livestock in both Madagascar and Tanzania, supporting previous evidence for mixed infections in small mammals (Moseley *et al.*, 2018). Horizontal genetic transfer plays an important role in the evolution and serological classification of *Leptospira* (Llanes *et al.*, 2016), and mixed infections within the same host provide the ideal environment for this to take place. Moreover, for *Borrelia*, it has been proposed that mixed infections may facilitate the maintenance of infection in reservoir hosts (Andersson *et al.*, 2013). Where serological diagnostic assays, such as MAT, rely on evaluating serological response to specific antigens, mixed infections with strains which may represent different serovars could complicate interpretation. Further research is needed to clarify the role of mixed infections in the evolution and epidemiology of *Leptospira*.

Our results emphasize that *Leptospira* epidemiology in tropical, developing country contexts, where close human contact with livestock is more likely and farming systems may promote contact between small mammals and livestock, may be very different to developed country settings. In addition, considering sampling methodology is important when comparing studies and planning surveillance and further work is needed to optimize abattoir sampling strategies. In Madagascar and other developing countries, evidence that livestock is infected with potentially novel *Leptospira* strains highlights the need for understanding the diversity of *Leptospira* circulating in livestock to inform diagnostic antigen panels and vaccine development in these regions.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0031182019001252.

Acknowledgements. We thank the staff of the Plague Central Laboratory, Institut Pasteur de Madagascar, for technical assistance, especially Fehivola Andriamiarimanana, as well as staff from the abattoirs for their assistance during sampling.

Financial support. This work was supported the Wellcome Trust (Senior Fellowship no. 095171 to S.T., Veterinary Training Fellowship no. 096400/Z/11/Z to K.A., Institutional Strategic Support Fund to the University of Aberdeen no. 2014815/Z/16/Z), the Biotechnology and Biological Sciences Research Council (no. BB/M010996/1), the University of Aberdeen Environment and Food Security theme and Institut Pasteur Madagascar.

Conflict of interest. None.

Ethical standards. Not applicable.

References

Allan KJ, Halliday JEB, Moseley M, Carter RW, Ahmed A, Goris MGA, Hartskeerl RA, Keyyu J, Kibona T, Maro VP, Maze MJ, Mmbaga BT, Tarimo R, Crump JA and Cleaveland S (2018) Assessment of animal hosts of pathogenic *Leptospira* in northern Tanzania. *PLoS Neglected Tropical Diseases* **12**, e0006444.

Andersson M, Scherman K and Råberg L (2013) Multiple-strain infections of *Borrelia afzelii*: a role for within-host interactions in the maintenance of antigenic diversity? *The American Naturalist* **181**, 545–554.

Bulach DM, Zuerner RL, Wilson P, Seemann T, McGrath A, Cullen PA, Davis J, Johnson M, Kuczek E, Alt DP, Peterson-Burch B, Coppel RL, Rood JI, Davies JK and Adler B (2006) Genome reduction in *Leptospira borgpetersenii* reflects limited transmission potential. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 14560–14565.

Costa F, Hagan JE, Calcagno J, Kane M, Torgerson P, Martinez-silveira MS, Stein C, Abela-ridder B and Ko AI (2015) Global morbidity and mortality of leptospirosis: a systematic review. *PLoS Neglected Tropical Diseases* **9**, e0003898.

https://doi.org/10.1017/S0031182019001252 Published online by Cambridge University Press

Dietrich M, Wilkinson DA, Soarimalala V, Goodman SM, Dellagi K and Tortosa P (2014) Diversification of an emerging pathogen in a biodiversity hotspot: *Leptospira* in endemic small mammals of Madagascar. *Molecular Ecology* **23**, 2783–2796.

Guedes IB, de Araújo SAA, de Souza GO, de Souza Silva SO, Taniwaki SA, Cortez A, Brandão PE and Heinemann MB (2019) Circulating *Leptospira* species identified in cattle of the Brazilian Amazon. *Acta Tropica* **191**, 212–216.

Guernier V, Lagadec E, Cordonin C, Le Minter G, Gomard Y, Pagès F, Jaffar-Bandjee M-C, Michault A, Tortosa P and Dellagi K (2016) Human leptospirosis on Reunion Island, Indian Ocean: are rodents the (Only) ones to blame? *PLOS Neglected Tropical Diseases* **10**, e0004733.

Halliday J, Daborn C, Auty H, Mtema Z, Lembo T, Bronsvoort BMD, Handel I, Knobel D, Hampson K and Cleaveland S (2012) Bringing together emerging and endemic zoonoses surveillance: shared challenges and a common solution. *Philosophical Transactions of the Royal Society B: Biological Sciences* **367**, 2872–2880.

Hamond C, Pestana CP, Medeiros MA and Lilenbaum W (2015) Genotyping of *Leptospira* directly in urine samples of cattle demonstrates a diversity of species and strains in Brazil. *Epidemiology and Infection* **144**, 72–75.

ILRI (2012) *Mapping of Poverty and Likely Zoonoses Hotspots. Zoonoses Report* 4. *Report to Department for International Development, UK.* International Livestock Research Institute, Nairobi, Kenya.

Kumar S, Stecher G and Tamura K (2016) MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution* **33**, 1870–1874.

Llanes A, Restrepo CM and Rajeev S (2016) Whole genome sequencing allows better understanding of the evolutionary history of *Leptospira interrogans* serovar *hardjo*. *PLoS ONE* **11**, 1–12.

Marshall RB and Chereshsky A (1996) Vaccination of dairy cattle against leptospirosis as a means of preventing human infections. *Surveillance* **23**, 27–28.

Maudlin I, Eisler MC and Welburn SC (2009) Neglected and endemic zoonoses. *Philosophical Transactions of the Royal Society B: Biological Sciences* **364**, 2777–2787.

Merien F, Portnoi D, Bourhy P, Charavay F, Berlioz-Arthaud A and Baranton G (2005) A rapid and quantitative method for the detection of *Leptospira* species in human leptospirosis. *FEMS Microbiology Letters* **249**, 139–147.

Mgode GF, Machang'u RS, Mhamphi GG, Katakweba A, Mulungu LS, Durnez L, Leirs H, Hartskeerl RA and Belmain SR (2015) *Leptospira* serovars for diagnosis of leptospirosis in humans and animals in Africa: common *Leptospira* isolates and reservoir hosts. *PLoS Neglected Tropical Diseases* **9**, e0004251.

Moseley M, Rahelinirina S, Rajerison M, Garin B, Piertney S and Telfer S (2018) Mixed *Leptospira* infections in a diverse reservoir host community, Madagascar, 2013–2015. *Emerging Infectious Diseases* **24**, 1137–1139.

Pagès F, Kuli B, Moiton M-PP, Goarant C and Jaffar-Bandjee M-CC (2015) Leptospirosis after a stay in Madagascar. *Journal of Travel Medicine* **22**, 136–139.

Picardeau M (2017) Virulence of the zoonotic agent of leptospirosis: still terra incognita? *Nature Reviews Microbiology* **15**, 297–307.

Rahelinirina S, Léon A, Harstskeerl RA, Sertour N, Ahmed A, Raharimanana C, Ferquel E, Garnier M, Chartier L, Duplantier J-M-M, Rahalison L, Cornet M and Harstkeerl RA (2010) First isolation and direct evidence for the existence of large small-mammal reservoirs of *Leptospira* sp. in Madagascar. *PLoS ONE* **5**, e14111.

Ratsitorahina M, Rahelinirina S, Michault A, Rajerison M, Rajatonirina S and Richard V (2015) Has Madagascar lost its exceptional leptospirosis free-like Status? *PLoS ONE* **10**, e0122683.

Smythe LD, Smith IL, Smith GA, Dohnt MF, Symonds ML, Barnett LJ and McKay DB (2002) A quantitative PCR (TaqMan) assay for pathogenic *Leptospira* spp. *BMC Infectious Diseases* **2**, 13.

Sonada RB, de Azevedo SS, Soto FRM, da Costa DF, de Morais ZM, de Souza GO, Gonçales AP, Miraglia F and Vasconcellos SA (2018) Efficacy of leptospiral commercial vaccines on the protection against an autochtonous strain recovered in Brazil. *Brazilian Journal of Microbiology* **49**, 347–350.

Taylor LH, Latham SM and Woolhouse MEJ (2001) Risk factors for human disease emergence. *Philosophical Transactions of the Royal Society B: Biological Sciences* **356**, 983–989.

Thornley CN, Baker MG, Weinstein P and Maas EW (2002) Changing epidemiology of human leptospirosis in New Zealand. *Epidemiology and Infection* **128**, 29–36.

Victoria B, Ahmed A, Zuerner RL, Ahmed N, Bulach DM, Quinteiro J, Harstkeerl RA and Hartskeerl RA (2008) Conservation of the S10-spc-a locus within otherwise highly plastic genomes provides phylogenetic insight into the genus *Leptospira*. *PLoS ONE* **3**, e2752.

Woolhouse ME and Gowtage-Sequeria S (2005) Host range and emerging and reemerging pathogens emerging infectious diseases. *Emerging Infectious Diseases* **11**, 1842–1847.

https://doi.org/10.1017/S0031182019001252 Published online by Cambridge University Press