Title: Insight into motility-dependent pathogenicity of the zoonotic spirochete

Leptospira

Short title: Motility-dependent spirochetal pathogenicity

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Abstract

Bacterial motility is crucial for many pathogenic species in the process of invasion and/or dissemination. The spirochete bacteria *Leptospira* spp. cause symptoms, such as hemorrhage, jaundice, and nephritis, in diverse mammals including humans. Although loss-of-motility attenuate the spirochete, the mechanism of the motility-dependent pathogenicity is unknown. Here, focusing on that *Leptospira* spp. swim in liquid and crawl on solid surfaces, we investigated the spirochetal dynamics on the host tissues by infecting cultured kidney cells from various species with pathogenic and nonpathogenic leptospires. We found that, in the case of the pathogenic leptospires, a larger fraction of bacteria attached to the host cells and persistently traveled long distances using the crawling mechanism. Our results associate the kinetics and kinematic features of the spirochetal pathogens with their virulence.

One Sentence Summary: Adhesivity and crawling motility over host tissue surfaces are closely related to the pathogenicity of a zoonotic spirochete.

Introduction

Many bacteria utilize motility to explore environments for survival and prosperity. For pathogenic species, the motility is a virulence factor, harming the host animal or plant health(6). For example, *Helicobacter pylori* requires motility to migrate towards the epithelial tissue in the stomach(7), and motility and chemotaxis are key factors that guide host invasion in different *Salmonella* serovars (8, 9). Movement and adhesion of the Lyme disease spirochete *Borrelia burgdorferi* in blood vessels are thought to be important during the process of host cell invasion(10). In the enteric pathogens, such as the enteropathogenic *Escherichia coli* (EPEC), the motility

machinery flagella are also important for adhesion to the host intestinal epithelium(11).

In this study, we address the association of bacterial motility with pathogenicity in the worldwide zoonosis leptospirosis. The causative agent of leptospirosis *Leptospira* spp. are Gram-negative bacteria belonging to the phylum Spirochaetes. The genus *Leptospira* is comprised of pathogenic, intermediate, and nonpathogenic species, which are classified into over 300 serovars defined based on the structural diversity of lipopolysaccharide (LPS)(2, 12). The pathogenic species affect various mammalian hosts such as livestock (cattle, pigs, horses, and others), companion animals (dogs and others), and humans, causing severe symptoms, such as hemorrhage, jaundice, and nephritis in some host-serovar pairs(2–4). The leptospire can be maintained in the renal tubules of recovered animals or reservoir hosts, and the urinary shedding of leptospire to the environment leads to infection in humans and other animals through contact with contaminated soil or water. Although the pathogenic mechanism of leptospirosis is not well elucidated, in addition to the pathogen-specific proteins such as Loa22(13), their motility using two periplasmic flagella (PFs) beneath the outer cell membrane (Fig. 1A) is known to be somehow involved in the infection and pathogenicity based on studies in animal models that have shown attenuation of the spirochetes by loss-of-motility(14, 15).

The rotation of the PFs in *Leptospira* gyrates both ends of the cell body and rotates the coiled cell body, allowing the spirochete not only to swim in fluids but also to crawl over solid surfaces(16, 17). Adherence and entry of pathogenic leptospire in the conjunctival epithelium(4) and in the paracellular routes of hepatocytes(18) were previously observed using scanning electron microscopy, suggesting adhesion to the host tissue surfaces and subsequent crawling of pathogenic leptospire. To verify this hypothesis, assuming the transition of leptospire between swimming and adhesion states

and between adhesion and crawling states in the equilibrium (Fig. 1B), we investigated the adhesion and crawling motility of *Leptospira* on the cultured kidney cells of various mammalian species.

Results

Steady-state analysis of *Leptospira* on the kidney cells. We infected cultured kidney cells from six different host species (rat, dog, monkey, mouse, cow, and human) with three *Leptospira* strains (the pathogenic *L. interrogans* serovars Icterohaemorrhagiae and Manilae, and the nonpathogenic *L. biflexa* serovar Patoc) expressing green fluorescent protein (GFP) within a chamber slide (Fig. 2A). We observed the *Leptospira* cells by epi-fluorescent microscopy (Fig. 2B) and measured the fractions of swimming [S], adhered [A], and crawling [C] bacteria on the kidney cells (Fig. 2C). Figs. 2D-E show that almost half of the pathogenic population transited from the swimming state to the adhesion and crawling states, whereas >75% non-pathogenic leptospires remained in the swimming state. We calculated the equilibrium constant between swimming and adhesion states (K_{S-A}) and between adhesion and crawling states (K_{A-C}) from the cell fractions using $K_{S-A} = [A] / [S]$ and $K_{A-C} = [C] / [A]$, respectively. The pathogenic leptospires had a significantly larger K_{A-C} in comparison with the nonpathogenic strain ($P < 0.05$); K_{S-A} did not seem to correlate to virulence (Fig. 2F). These thermodynamic parameters suggest that the biased transition from adhesion to crawling would be responsible for the virulence of *Leptospira* (Fig. 2G).

Crawling motility. Taking the results of the steady-state analysis, we focused on the crawling motility of individual *Leptospira* cells on the kidney cells. Although the crawling speed varied among the measured host-bacterium pairs, *L. interrogans* serovar Icterohaemorrhagiae showed

significantly faster speed than the others, indicating the species/serovar dependence of the crawling ability (Fig. 3A). On the other hand, there is no difference in crawling speed between *L. interrogans* serovar Manilae and *L. biflexa* serovar Patoc, suggesting that the crawling speed itself is not related to leptospiral virulence. Meanwhile, we observed that some leptospiral cells attached to the kidney cells and moved smoothly for periods, migrating over long-distances (upper panels of Figs. 3B-C; Movie S2), whereas others frequently reversed the crawling direction (lower panels of Figs. 3B-C; Movie S3). Cell movements with reversals are considered to be diffusive and thus can be evaluated by plotting the mean square displacement (MSD) against time, a general methodology for diffusion (Brownian motion) analysis(19). The MSD of simple diffusion without directivity is proportional to time, and therefore double-logarithmic MSD plots from such non-directional diffusion represent slopes of ~ 1 , whereas those from directive movements show MSD slopes of ~ 2 , representing the relatively long distance traveled by the cells (Fig. S1). Double-logarithmic MSD plots obtained from each individual leptospire showed a wide range of MSD slopes (example data are shown in Fig. 3D) and differed for each host-*Leptospira* pair (Fig. 3E left and Fig. S2). The nonpathogenic strain showed the slope of ~ 1 , while the pathogenic strains had significantly larger slopes that denote directive motion (Fig. 3E right). Thus, concerning the crawling motility, directivity and persistency rather than speed could be crucial for virulence.

Discussion

Our results suggested the importance of adhesion to and persistent crawling on the host tissue for the pathogenicity of *Leptospira*. The thermodynamic and kinematic parameters are associated in Fig 4A, showing the tendency that pathogenic species are biased to the crawling state and can migrate longer

distance on the host tissue surfaces. The crawling motility of *Leptospira* is caused by the attachment of the spirochete cell body to surfaces via adhesive cell surface components(16, 20). The successive alternation in the attachment and detachment of adhesins allows for this progressive movement by the spirochetes, however, an excessively strong adhesion can inhibit crawling(16). LPS, the molecular basis for the identification of the different *Leptospira* serovars(2, 12), is thought to be a crucial adhesin important for this crawling motion(16). Thus, it is possible that compatibility of the serological characteristics of leptospires and the surface properties of the host tissue might affect the crawling behavior over the tissue surfaces and the subsequent clinical consequences. The results of our biophysical experiments outline a plausible framework for the adhesion and crawling-dependent pathogenicity of *Leptospira* (Fig. 4B). The biased transition from the adhesion to the crawling state and the long-distance, persistent crawling allows leptospires to explore the host's cell surfaces, increasing the probability of encountering routes for invasion through their intracellular tight junctions (Fig. 4B, left). In contrast, the swimming or weakly attached leptospires can be swept by external forces, such as intermittent urination, and diffusive crawling by which leptospires cannot be disseminated over host tissues will not be involved in invasion (Fig. 4B, right).

Some bacterial pathogens are specialized to invade a very limited array of hosts, whereas others can infect multiple host species. The host range differs for each pathogen and the clinical symptoms depend on each host-pathogen combination. The same applies for leptospirosis, the outcome of *Leptospira* infection depends on the host-serovar association, and some animal species can become an asymptomatic reservoir for particular *Leptospira* serovars. The present experiments also provided data allowing us to discuss the host dependence of the leptospiral dynamics. Among the investigated materials, serovar Manilae vs rats and serovar Icterohaemorrhagiae vs rats are typically

asymptomatic pairs, and Fig. 4A shows that the pairs with reservoirs have lower scores in comparison with those causing severe symptoms, such as Manilae vs humans, Icterohaemorrhagiae vs dogs, and others. This implies that the surface dynamics of the spirochete could be related to their host-dependent pathogenicity. Understanding the mechanism of the host preferences by pathogens is important for prevention of the infection spread, but the host-pathogen association in leptospirosis is ambiguous. Also, microarray analyses have revealed that regulation of gene expression in *Leptospira* is affected by its interaction with host cells(21, 22). Although host immune responses against *Leptospira* infection remain mostly unknown, pathogenic leptospires interfere with the complement system through the degradation of complement proteins(23) and inhibition of coagulation via binding to thrombin(24). Thus, abundant factors in both bacteria and hosts should be investigated for a deeper understanding of the host-dependent pathogenicity.

Materials and Methods

***Leptospira* strains and growth conditions.** Pathogenic serovars of *L. interrogans* serovar Icterohaemorrhagiae (strain WFA135), an isolate from *Rattus norvegicus* in Tokyo, Japan, serovar Manilae (strain UP-MMC-NIID)(25, 26) and a saprophytic *L. biflexa* serovar Patoc (strain Patoc I) were used in this study. The serovar of WFA135 was determined by multiple loci variable number of tandem repeats analysis (MLVA) and DNA sequencing of the *lic12008* gene(26, 27). Bacteria were cultured in enriched Ellinghausen-McCullough-Johnson-Harris (EMJH) liquid medium (BD Difco, NJ, USA) containing 25 µg/mL spectinomycin at 30 °C for 2 (*L. biflexa*) or 4 (*L. interrogans*) days until the stationary phase. To track *Leptospira* cells when in co-culture with mammalian kidney cells, a green fluorescent protein (GFP) was constitutively expressed in each strain (Fig. 1D; Movie S1).

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150 **Mammalian cells and media.** Mammalian kidney epithelial cell lines used included MDCK-NBL2
 151 (dog), NRK-52E (rat), Vero (monkey), MDBK-NBL1 (cow), TCMK-1 (mouse), and HK-2 (human).
 152 MDCK, Vero, TCMK, and MDBK cells were maintained in Eagle's minimum essential media
 153 (MEM) (Sigma-Aldrich, Darmstadt, Germany) containing 10% fetal bovine serum or 10% horse
 154 serum (Nacalai Tesque, Kyoto, Japan) at 37 °C and 5% CO₂. NRK cells were maintained in
 155 Dulbecco's modified Eagle's media (DMEM) (Thermo Fisher Scientific, MA, USA) with 4 mM
 156 L-glutamine, adjusted to contain 1.5 g/L sodium bicarbonate and 4.5 g/L glucose, and with 5%
 157 bovine calf serum (Nacalai Tesque) at 37 °C and 5% CO₂. HK-2 cells were maintained in
 158 keratinocyte serum-free (KSF) media with 0.05 mg/mL bovine pituitary extract and 5 ng/mL human
 159 recombinant epidermal growth factor (Gibco - Thermo Fisher Scientific, MA, USA). All culture
 160 conditions contained a 5% antibiotic / antimycotic mixed solution (Nacalai Tesque). Cells were
 161 treated with a 0.1% trypsin - EDTA solution (Nacalai Tesque) to dislodge the cells during each
 162 passage process.

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164 **GFP expression in *L. interrogans* and *L. biflexa*.** For the construction of a replicable plasmid in *L.*
 165 *interrogans*, the corresponding *rep-parB-parA* region of the plasmid pGui1 from the *L. interrogans*
 166 serovar Canicola strain Gui44 plasmid pGui1(28) was amplified from a *L. interrogans* serogroup
 167 Canicola isolate, and the amplified product was cloned into the PCR-generated pCjSpLe94(29) by
 168 NEBuilder HiFi DNA Assembly cloning (New England BioLabs), generating pNKLiG1. The *flgC*
 169 promoter region and *gfp* were amplified from pCjSpLe94 and pAcGFP1 (Clontech), respectively,
 170 and the amplified products were cloned into the *SalI*-digested pNKLiG1 for *L. interrogans* or the

SalI-digested pCjSpLe94 for *L. biflexa*. The plasmids were transformed into strains WFA135, UP-MMC-NIID or Patoc I by conjugation with *E. coli* β 2163 harboring the plasmid(30). Primer sequences used in this study are listed in Table S1. Expression of GFP did not affect motility in the *Leptospira* serovars (Fig. S3).

Preparation of kidney cells and *Leptospira* cells in a chamber slide. Kidney cells were harvested with 0.1% trypsin and 0.02% EDTA in a balanced salt solution (Nacalai Tesque) and plated onto a chamber slide (Iwaki, Tokyo, Japan) using their corresponding media without antibiotics. The slides were incubated for 48 h until a monolayer was formed and washed twice with media to remove non-adherent cells. The cells were incubated for a further 2 h at 37 °C and 5% CO₂. Approximately 500 ml of stationary phase *Leptospira* cells were harvested by centrifugation at 1,000 g for 10 min at room temperature, washed twice in PBS, then resuspended in the corresponding kidney cell culture media without antibiotics at 37 °C to a concentration of 10⁷ cells / ml. These suspensions (1 ml) were then added into the corresponding chamber slides containing the kidney cell layer, and the chamber slides were incubated at 37 °C for 1 h.

Microscopy observation and adhesion-crawling assay. The movement behaviors, swimming, adhesion and crawling of the *Leptospira* cells on the kidney cells were observed using a dark-field microscope (BX53, Splan 40×, NA 0.75, Olympus, Tokyo, Japan) with an epi-fluorescent system (U-FBNA narrow filter, Olympus) and recorded by a CCD-camera (WAT- 910HX, Watec Co., Yamagata, Japan) at 30 frames per second. *Leptospira* cells were tracked using an ImageJ (NIH, MD, USA)-based tracking system and the motion parameters such as motile fraction, velocity and MSD

were analyzed using Excel-based VBA (Microsoft, WA, USA). The two-dimensional MSD of individual leptospiral cells during a period Δt was calculated by the following equation: $MSD(\Delta t) = \langle (x_{i+\Delta t} - x_i)^2 + (y_{i+\Delta t} - y_i)^2 \rangle$, where (x_i, y_i) is the bacterial position at I (see also Supplementary Fig. 1).

Statistical analysis. All experiments were performed in triplicate. Statistical differences between data were evaluated using a Student's t -test. The data clustering was performed independently for each experiment using the k-means method in OriginPro (OriginLab Corp., MA, USA). The clustering method grouped the data population into a specified number of clusters referring to the Euclidian distance of each data point from the centroid of the cluster, calculated at every clustering process, and reclassifying the data point to the nearest cluster.

Supplementary Materials

Tables S1. Primer sequences used in this study

Fig. S1. Explanation of MSD plot.

Fig. S2. Histograms of the MSD slopes

Fig. S3. Effect of GFP expression on the *Leptospira* motility.

Movie S1. Epi-fluorescent images of *L. interrogans* on the rat kidney cell

Movie S2. Progressive, long-distance crawling of *L. interrogans* on the monkey kidney cells

Movie S3. Crawling of *L. interrogans* with highly frequent reversal on the dog kidney cells

References

- 215 1. A. Bäumler, F. C. Fang, Host specificity of bacterial pathogens. *Cold Spring Harb. Perspect. Med.* **3** (2013),
216 doi:10.1101/cshperspect.a010041.
- 217 2. M. Picardeau, Virulence of the zoonotic agent of leptospirosis: still terra incognita? *Nat. Rev. Microbiol.* **15**,
218 297–307 (2017).
- 219 3. B. Adler, A. de la Peña Moctezuma, *Leptospira* and leptospirosis. *Vet. Microbiol.* **140**, 287–296 (2010).
- 220 4. A. R. Bharti, J. E. Nally, J. N. Ricaldi, M. A. Matthias, M. M. Diaz, M. A. Lovett, P. N. Levett, R. H. Gilman,
221 M. R. Willig, E. Gotuzzo, J. M. Vinetz, Peru-United States Leptospirosis Consortium, Leptospirosis: a
222 zoonotic disease of global importance. *Lancet Infect. Dis.* **3**, 757–771 (2003).
- 223 5. P. N. Levett, Leptospirosis. *Clin. Microbiol. Rev.* **14**, 296–326 (2001).
- 224 6. C. Josenhans, S. Suerbaum, The role of motility as a virulence factor in bacteria. *Int. J. Med. Microbiol.* **291**,
225 605–614 (2002).
- 226 7. M. Clyne, T. Ocroinin, S. Suerbaum, C. Josenhans, B. Drumm, Adherence of isogenic flagellum-negative
227 mutants of *Helicobacter pylori* and *Helicobacter mustelae* to human and ferret gastric epithelial cells. *Infect.*
228 *Immun.* **68**, 4335–4339 (2000).
- 229 8. J. E. Olsen, K. H. Hoegh-Andersen, J. Casadesús, J. T. Rosenkrantz, M. S. Chadfield, L. E. Thomsen, The
230 role of flagella and chemotaxis genes in host pathogen interaction of the host adapted *Salmonella enterica*
231 serovar Dublin compared to the broad host range serovar *S. Typhimurium*. *BMC Microbiol.* **13**, 67 (2013).
- 232 9. A. Siitonen, M. Nurminen, Bacterial motility is a colonization factor in experimental urinary tract infection.
233 *Infect. Immun.* **60**, 3918–3920 (1992).
- 234 10. R. Ebady, A. F. Niddam, A. E. Boczula, Y. R. Kim, N. Gupta, T. T. Tang, T. Odisho, H. Zhi, C. A. Simmons, J.
235 T. Skare, T. J. Moriarty, Biomechanics of *Borrelia burgdorferi* vascular interactions. *Cell Rep.* **16**, 2593–2604
236 (2016).

- 237 11. C. P. Cheney, P. A. Schad, S. B. Formal, E. C. Boedeker, Species specificity of *in vitro* *Escherichia coli*
238 adherence to host intestinal cell membranes and its correlation with *in vivo* colonization and infectivity. *Infect.*
239 *Immun.* **28**, 1019–1027 (1980).
- 240 12. G. M. Cerqueira, M. Picardeau, A century of *Leptospira* strain typing. *Infect. Genet. Evol. J. Mol. Epidemiol.*
241 *Evol. Genet. Infect. Dis.* **9**, 760–768 (2009).
- 242 13. P. Ristow, P. Bourhy, F. W. da Cruz McBride, C. P. Figueira, M. Huerre, P. Ave, I. S. Girons, A. I. Ko, M.
243 Picardeau, The OmpA-like protein Loa22 is essential for leptospiral virulence. *PLoS Pathog.* **3**, e97 (2007).
- 244 14. A. Lambert, M. Picardeau, D. A. Haake, R. W. Sermswan, A. Srikrum, B. Adler, G. A. Murray, FlaA proteins
245 in *Leptospira interrogans* are essential for motility and virulence but are not required for formation of the
246 flagellum sheath. *Infect. Immun.* **80**, 2019–2025 (2012).
- 247 15. E. A. Wunder, C. P. Figueira, N. Benaroudj, B. Hu, B. A. Tong, F. Trajtenberg, J. Liu, M. G. Reis, N. W.
248 Charon, A. Buschiazzi, M. Picardeau, A. I. Ko, A novel flagellar sheath protein, FcpA, determines filament
249 coiling, translational motility and virulence for the *Leptospira* spirochete. *Mol. Microbiol.* **101**, 457–470
250 (2016).
- 251 16. H. Tahara, K. Takabe, Y. Sasaki, K. Kasuga, A. Kawamoto, N. Koizumi, S. Nakamura, The mechanism of
252 two-phase motility in the spirochete *Leptospira*: Swimming and crawling. *Sci. Adv.* **4**, eaar7975 (2018).
- 253 17. P. J. Cox, G. I. Twigg, Leptospiral motility. *Nature.* **250**, 260–261 (1974).
- 254 18. S. Miyahara, M. Saito, T. Kanemaru, S. Y. A. M. Villanueva, N. G. Gloriani, S. Yoshida, Destruction of the
255 hepatocyte junction by intercellular invasion of *Leptospira* causes jaundice in a hamster model of Weil's
256 disease. *Int. J. Exp. Pathol.* **95**, 271–281 (2014).
- 257 19. A. Kusumi, Y. Sako, M. Yamamoto, Confined lateral diffusion of membrane receptors as studied by single
258 particle tracking (nanovid microscopy). Effects of calcium-induced differentiation in cultured epithelial cells.

- 259 *Biophys. J.* **65**, 2021–2040 (1993).
- 260 20. N. W. Charon, C. W. Lawrence, S. O'Brien, Movement of antibody-coated latex beads attached to the
261 spirochete *Leptospira interrogans*. *Proc. Natl. Acad. Sci. U. S. A.* **78**, 7166–7170 (1981).
- 262 21. K. Satou, M. Shimoji, H. Tamotsu, A. Juan, N. Ashimine, M. Shinzato, C. Toma, T. Nohara, A. Shiroma, K.
263 Nakano, K. Teruya, Y. Terabayashi, S. Ohki, N. Koizumi, S. Okano, T. Suzuki, T. Hirano, Complete genome
264 sequences of low-passage virulent and high-passage avirulent variants of pathogenic *Leptospira interrogans*
265 serovar Manilae strain UP-MMC-NIID, originally isolated from a patient with severe leptospirosis,
266 determined using PacBio single-molecule real-time technology. *Genome Announc.* **3** (2015),
267 doi:10.1128/genomeA.00882-15.
- 268 22. C. Toma, G. L. Murray, T. Nohara, M. Mizuyama, N. Koizumi, B. Adler, T. Suzuki, Leptospiral outer
269 membrane protein LMB216 is involved in enhancement of phagocytic uptake by macrophages. *Cell.*
270 *Microbiol.* **16**, 1366–1377 (2014).
- 271 23. T. R. Fraga, D. D. S. Courrol, M. M. Castiblanco-Valencia, I. Y. Hirata, S. A. Vasconcellos, L. Juliano, A. S.
272 Barbosa, L. Isaac, Immune evasion by pathogenic *Leptospira* strains: the secretion of proteases that directly
273 cleave complement proteins. *J. Infect. Dis.* **209**, 876–886 (2014).
- 274 24. L. G. Fernandes, Z. M. de Moraes, S. A. Vasconcellos, A. L. T. O. Nascimento, *Leptospira interrogans*
275 reduces fibrin clot formation by modulating human thrombin activity via exosite I. *Pathog. Dis.* **73** (2015),
276 doi:10.1093/femspd/ftv001.
- 277 25. N. Koizumi, H. Watanabe, Leptospiral immunoglobulin-like proteins elicit protective immunity. *Vaccine.* **22**,
278 1545–1552 (2004).
- 279 26. L. A. Santos, H. Adhikarla, X. Yan, Z. Wang, D. E. Fouts, J. M. Vinetz, L. C. J. Alcantara, R. A. Hartskeerl,
280 M. G. A. Goris, M. Picardeau, M. G. Reis, J. P. Townsend, H. Zhao, A. I. Ko, E. A. Wunder, Genomic

comparison among global isolates of *L. interrogans* serovars Copenhageni and Icterohaemorrhagiae identified natural genetic variation caused by an Indel. *Front. Cell. Infect. Microbiol.* **8**, 193 (2018).

27. N. Koizumi, H. Izumiya, J.-J. Mu, Z. Arent, S. Okano, C. Nakajima, Y. Suzuki, M. Mizutani Muto, T. Tanikawa, K. R. Taylor, N. Komatsu, K. Yoshimatsu, H. Thi Thu Ha, M. Ohnishi, Multiple-locus variable-number tandem repeat analysis of *Leptospira interrogans* and *Leptospira borgpetersenii* isolated from small feral and wild mammals in East Asia. *Infect. Genet. Evol. J. Mol. Epidemiol. Evol. Genet. Infect. Dis.* **36**, 434–440 (2015).

28. W.-N. Zhu, L.-L. Huang, L.-B. Zeng, X.-R. Zhuang, C.-Y. Chen, Y.-Z. Wang, J.-H. Qin, Y.-Z. Zhu, X.-K. Guo, Isolation and characterization of two novel plasmids from pathogenic *Leptospira interrogans* serogroup Canicola serovar Canicola strain Gui44. *PLoS Negl. Trop. Dis.* **8**, e3103 (2014).

29. M. Picardeau, Conjugative transfer between *Escherichia coli* and *Leptospira* spp. as a new genetic tool. *Appl. Environ. Microbiol.* **74**, 319–322 (2008).

30. L. Slamti, M. Picardeau, Construction of a library of random mutants in the spirochete *Leptospira biflexa* using a *mariner* transposon. *Methods Mol. Biol. Clifton NJ.* **859**, 169–176 (2012).

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Author contributions J.X., N.K. and S.N. planned the project; J.X and N.K. carried out the

experiments; S.N. set up the optical system and programs for data analysis; J.X. and S.N. analyzed the data; J.X., N.K. and S.N. wrote the paper.

Competing interests The authors declare that they have no competing interests.

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Data availability The data supporting the findings of this study are available from the corresponding author upon request.

Fig. 1. Structure of *Leptospira* and working model. (A) Schematic diagram of the *Leptospira* cell structure. (B) A three-state kinetic model assuming a transition between “swimming” (floating above cell layers without physical contact to the cells) and “adhesion” (attachment to the cell layer without migration), and between “adhesion” and “crawling” (attachment to the cell layer and movement over surfaces), with K_{S-A} and K_{A-C} represent the equilibrium constants of each transition, respectively.

Fig. 2. Steady-state motility and adhesion of *Leptospira* cells on kidney cells. (A) Schematic of the motility assay on the cultured kidney cells by epi-fluorescent microscopy. Fully confluent kidney cells from either animals or humans were cultured in an observation chamber, and *Leptospira* cells that constitutively expressed GFP were added to the cultures. (B) Example of an epi-fluorescence image of the *L. interrogans* serovar Icterohaemorrhagiae, as indicated by the yellow triangles, on the rat kidney cell line, NRK-52E. The nucleus of the kidney cell is shown by the white arrow (Movie S1). (C) Image sequences of swimming (top), adhered (middle), and crawling (bottom) leptospiral

cells. Yellow and white arrows indicate *Leptospira* cells and kidney cells, respectively. No kidney cells were observed due to out of focus during the measurement of *Leptospira* cells swimming in liquid media. (D) Ternary plot of the cell fractions in a state of swimming, adhesion, or crawling. The inset schematically explains how to read the ternary plot using an example plot with 30% for I, 45% for II, and 25% for III. Legend is shown to the right of the ternary plot, and M, I and P indicate the *L. interrogans* serovar Manilae, *L. interrogans* serovar Icterohaemorrhagiae, and *L. biflexa* serovar Patoc, respectively. Each data point represents the mean of triplicate experiments and ~ 2,400 leptospiral cells were measured per host-serovar pair. (E) Average values of the cell fractions. Error bars are the standard deviation. (F) The host-bacterium dependence of the equilibrium constants K_{S-A} and K_{A-C} , calculated from the data shown in **D**; rat (R), dog (D), monkey (Mn), mouse (Mu), cow (C), and human (H). The averaged values determined for each bacterial strain are shown by horizontal lines and are plotted with the standard deviation in the right; *L. interrogans* Manilae (M), *L. interrogans* Icterohaemorrhagiae (I), and *L. biflexa* Patoc I (P). The statistical analysis was performed by Mann-Whitney U test (* $P < 0.05$). (G) Schematic explanation of the kinetic difference between pathogenic and non-pathogenic leptospires.

Fig. 3. Analysis of the crawling movement of *Leptospira* on kidney cell layers. (A) Average crawling speeds determined for each host-bacterium pair. More than 90 bacteria were measured for each pair. The averaged values calculated for each bacterial strain are shown by horizontal lines (left) and are plotted with the were (B) Examples of persistent crawling (upper panels) and diffusive crawling (lower panels) of *Leptospira* cells obtained by single-cell tracking in 10 s. Colors indicate time courses in the order of red orange yellow green blue, and indigo. (C) Example time courses of leptospires crawling on the kidney cell surfaces; inset, expanded view of cell displacements and schematics of motion patterns. The upper and lower panels show the long-distance migration

represented by directive crawling and the limited migration due to frequent reversal, respectively.

(D) Examples of MSD vs time plots, evaluating the directivity of individual leptospiral cell movements: Plots with slopes ~ 2 indicate persistently directive crawling (upper), whereas those with ~ 1 indicate a non-directional movement (lower), i.e., motion with high frequency of reversal patterns and short net migration distance (refer to the schematic explanation of motion pattern in insets of C). (E) MSD slopes determined for each host-bacterium pair. The boxes show the 25th (the bottom line), 50th (middle), and 75th (top) percentiles, and the vertical bars show the minimum and maximum values. The right panel show the strain-dependence of the medians. The statistical analysis was performed by Mann-Whitney U test (* $P < 0.05$).

Fig. 4. Correlation of adhesion, motility and pathogenicity. (A) Relationship between K_{A-C} and MSD slope (i.e., crawling persistency). The gray line is the result of quadratic curve fitting. The correlation coefficient is 0.77. See Fig. 2D for symbols. (B) A plausible model for crawling-dependent pathogenicity of *Leptospira*. In the cases that lead to severe symptoms (left), leptospiral cells were biased to the crawling state, and most of the crawling cells showed directional translation persistently over host tissue surfaces, increasing the invasion probability. For the asymptomatic or non-infectious cases, many leptospires remained in the swimming state and might be removed through body fluids or urination. Some fractions of the adhered leptospires were able to crawl, but their migration distances were limited due to frequent reversal.

Supplementary Materials

Title: Insight into motility-dependent pathogenicity of the zoonotic spirochete *Leptospira*

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This file includes:

Tables S1. Primer sequences used in this study

Fig. S1. Explanation of MSD plot.

Fig. S2. Histograms of the MSD slopes

Fig. S3. Effect of GFP expression on the *Leptospira* motility.

Other Supplementary Materials for this manuscript include the following:

Movie S1. Epi-fluorescent images of *L. interrogans* on the rat kidney cell

Movie S2. Progressive, long-distance crawling of *L. interrogans* on the monkey kidney cells

Movie S3. Crawling of *L. interrogans* with highly frequent reversal on the dog kidney cells

Table S1. Primer sequences used in this study

Target	Primer sequence (5'→3')	
	Forward	Reverse
<i>rep-parB-parA</i> (pGui1)	TCGACGCCGGCCAGCGTTACTTCATAGCATCTTGTTTC	GCTGGAGCTCCACCGGCTCGACTCTTACGGTGTGTTAG
pNKLIG1 (pCjSpLe94 derivative)	CGGTGGAGCTCCAGCTTTG	GCTGGCCGGCGTCGAAAAGTAAGCACCTGTTATTGC
<i>flgC</i> promoter	TATCGATACCGTCGACCCGAGCTTCAAGGAAGATTCCTA	ATGGAAACCTCCCTCATTTA
AcGFP	GAGGGAGGTTTCCATATGACCATGATTACGCCAAGC	TATCGATACCGTCGATCACTTGTACAGCTCATCCATG

Fig. S1. Explanation of MSD plot. (A) To explain the difference in MSD vs time plot of “directed movement” (Figs. 3B-C upper) from that of “diffusive movement” (Figs. 3B-C lower), we performed a computer simulation by considering a particle stepping to the left (-1) or to the right ($+1$) with the probability of P_{toL} and $P_{toR} (= 1 - P_{toL})$, respectively, in a time interval Δt . $P_{toL} = 0.5$ ($P_{toR} = 0.5$) and $P_{toL} = 0.1$ ($P_{toR} = 0.9$) were assumed for simulating simple diffusion and movement biased to the right, respectively, and the step direction was determined by a random number (rnd) from 0.0 to 1.0 generated in each event: If $rnd < P_{toL}$, the particle steps to the left ($+1$). The time course of the particle position was analyzed as shown in Methods. MSD vs time plots obtained by the simulation show that (B) simple diffusion and (C) directed movement give a linear line and a quadratic curve, respectively (left panels), therefore exhibiting linear lines with slopes of ~ 1 and ~ 2 in double-logarithmic plots (right panels). Red lines are regression lines fitted to data points obtained by simulation.

Fig. S2. Histograms of the MSD slopes. Dashed lines are the results of curve fitting.

Fig. S3. Effect of GFP expression on the *Leptospira* motility. No significant different found between the swimming speeds of GFP expressing strains and wild type strains in each serovar.