Adhesivity and motility of a zoonotic spirochete: Implications in host-dependent pathogenicity

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Bacterial pathogens can infect particular hosts or have a broad range of host species. Mechanisms of host preferences involve complicated host-pathogen associations such as bacterial adherence, gene regulation, and the host's immune responses[1]. Here, we show the role of adhesivity and motility of the zoonotic spirochete *Leptospira* in host-dependent pathogenicity. *Leptospira* spp. are classified into more than 300 serovars and whereas infection of susceptible mammals can cause clinical symptoms, it can also result in an asymptomatic colonization in some cases, thereby generating maintenance hosts[2–5]. Although the severity of leptospirosis is generally dependent on a combination of *Leptospira* serovars and host species, the scenarios leading to such diverse consequences of infection remain unclear. To investigate the underlying mechanisms of the different outcomes of *Leptospira* infection, we infected kidney cells with various prepared pairs of *Leptospira* serovars and analyzed the *Leptospira* dynamics on the host cells. Quantitatively monitoring the behavior of individual *Leptospira* cells showed that adherence and movement on the host cell surface, called "crawling" correlated with the severity of infection in the host. In the

most severe cases, a large fraction of leptospires attached to the host cells and persistently traveled long distances using the crawling mechanism. The biophysical implications of the kinetics and kinematic features of these bacterial pathogens were found to be critical factors for disease severity in their hosts.

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. For example, *Bacillus anthracis* primarily infects livestock and wild herbivores, transmitting to humans via direct or indirect contact with infected animals or animal products. However, its occurrence in carnivores is rare. Salmonellosis is caused by over 2000 serovars of *Salmonella enterica* and its transmission occurs from reptile reservoirs to a wide range of susceptible hosts including humans and other mammals. Virulence in the case of plant bacterial pathogens like *Actinobacteria* spp. and *Pantoea* spp. is host specific, and is believed to be related to the structure of the root microbiome and other factors that optimize their symbiosis (e.g., plant hormones, drought stress, temperature and salinity)[6,7]. In some cases, the mechanisms correlating host dependency to bacterial pathogenicity have been investigated. A correlation was found between the host range of the opportunistic food-poisoning bacterium *Listeria monocytogenes* and its host binding affinity through the listerial protein internalin A, which interacted with E-cadherin in intestinal epithelial cells thereby facilitating its invasion[8]. Here, we show that the adhesivity and motility of the zoonotic spirochete *Leptospira interrogans* are crucial factors responsible for their host-dependent pathogenicity.

Leptospirosis is a worldwide zoonosis, affecting various mammalian hosts such as livestock (cattle, pigs, horses, and others), companion animals (dogs and others), and humans, with consequences varying from asymptomatic to mild and fatal symptoms[2–4]. The causative agents *Leptospira* spp. are Gram-negative bacteria belonging to the phylum Spirochaetes. The genus *Leptospira* is comprised by over 300 serovars defined based on the structural diversity of lipopolysaccharide (LPS)[2,9]. Humans and other animals develop clinical disease; however, some animal species can become an asymptomatic reservoir for particular *Leptospira* serovars. Thus, the outcome of *Leptospira* infection depends on the

host-serovar association. Some host-serovar pairs can cause severe symptoms, such as hemorrhage, jaundice, and nephritis. In contrast, the same serovars can be maintained in the renal tubules of reservoir animals that will then intermittently shed leptospires in their urine over long periods[3,5]. The urinary shedding of leptospires to the environment leads to infection in humans and other animals through contact with contaminated soil or water. *Leptospira* spp. do not have an exotoxin-secretion machinery such as the type III secretion system[2]. The pathogenic *Leptospira*-specific protein Loa22 is known to be an essential virulence factor[10], but other proteins such as the fibronectin-binding protein LigB and the outer membrane protein LipL32 expressed solely in pathogenic strains, are not involved in their pathogenicity[11,12]. Despite these virulence factors being common among pathogenic *Leptospira* serovars, the host-dependent mechanisms of pathogenicity remain unclear.

Studies in animal models have shown that motility is an important factor involved in leptospiral virulence[13,14]. Motility is a critical component that facilitates infection for many species of bacteria[15]. *Helicobacter pylori* requires motility to migrate towards the epithelial tissue in the stomach[16] and motility and chemotaxis are key factors that guide host invasion in different *Salmonella* serovars [17,18]. In the case of Lyme disease, movement and adhesion by the spirochete *Borrelia burgdorferi* in blood vessels are thought to be important during the process of host cell invasion[19]. *Leptospira* spp. have two periplasmic flagella (PFs) beneath the outer cell membrane (Fig. 1A). The rotation of the PFs in the periplasmic space gyrates both ends of the cell body and rotates the coiled cell body allowing *Leptospira* not only to swim in fluids but also to crawl over solid surfaces[20,21]. Adherence and entry of pathogenic leptospires in the conjunctival epithelium[4] and in the paracellular routes of hepatocytes[22] were previously observed using scanning electron microscopy, raising the possibility that adhesion to the host tissue surfaces and subsequent surface movements are determinants of the host-dependent leptospiral pathogenicity. To verify this hypothesis, assuming the transition of leptospires between swimming and adhesion states and between adhesion and crawling states in the equilibrium (Fig. 1B), we quantitatively determined the adhesion and crawling motility of three *Leptospira* strains: the pathogenic *L. interrogans* serovars Icterohaemorrhagiae and Manilae and the nonpathogenic *L. biflexa* serovar Patoc on

93 cultured kidney cells from six different host species. We then discussed the correlation of the
94 leptospiral dynamics with host-dependent outcomes using statistical clustering based on the
95 measured parameters.

96 We first measured the fractions of swimming [S], adhered [A], and crawling [C]
97 *Leptospira* cells on the kidney cells (Figs. 1C-D and 2A), showing the dependence of
98 leptospiral dynamics on the host-serovar combination (Fig. 2B). We calculated the
99 equilibrium constant between swimming and adhesion states ($K_{S\text{-}A}$) and between adhesion
100 and crawling states ($K_{A\text{-}C}$) from the cell fractions using $K_{S\text{-}A}$ = [A] / [S] and $K_{A\text{-}C}$ = [C] / [A],
101 respectively (Fig. 2C). The data points scattered on the ternary plot of the cell fraction and
102 the pairwise plot of the equilibrium constants were statistically classified into three clusters.

103 Next, we measured the crawling speed of each individual cell on the kidney cells (Fig.
104 3A). Concerning the crawling motility, we observed that some leptospiral cells attached to
105 the kidney cells and moved smoothly for periods of time, migrating over long-distances (Figz.
106 3B, D, and F; Supplementary Movie 2), whereas others frequently reversed the crawling
107 direction (Figs. 3C, E, and G; Supplementary Movie 3). Cell movements with reversals are
108 considered to be diffusive and thus can be evaluated by plotting the mean square
109 displacement (MSD) against time, a general methodology for diffusion (Brownian motion)
110 analysis[23]. The MSD of simple diffusion without directivity is proportional to time, and
111 therefore double-logarithmic MSD plots from such non-directional diffusion represent slopes
112 of ~1, whereas those from directive movements show MSD slopes of ~2, representing the
113 relatively long distance travelled by the cells (Supplementary Fig. 1). Double-logarithmic
114 MSD plots obtained from each individual leptospiral cells showed a wide range of MSD
115 slopes and differed for each host-serovar pair (Figs. 3H-J and Supplementary Fig. 2). The
116 data of the crawling speeds and MSD slopes (i.e. crawling persistency) were also grouped
117 into statistical clusters.

118 To investigate the relevance of the measured parameters on the outcomes of leptospiral
119 infection, we translated Figs. 2–3 into Figs. 4A–D, where the mean values obtained for each
120 statistical cluster were indicated, and the serovar-host pairs were discriminated by their
121 clinical aspects, according to a literature search: red, pairs that exhibit any clinical signs;

yellow, isolation of leptospires from asymptomatic animals or natural reservoirs (i.e., rats); green, pairs containing the nonpathogenic *Leptospira* strain (i.e., non-infection); grey, no report (Table 1). Red, green and yellow pairs were mostly grouped together, although yellow pairs fluctuated between clusters with a red or green pair majority. Given that the determined clusters reflect the differences in outcomes of the host after infection by each *Leptospira* strain, our results suggest the involvement of adhesion and crawling in leptospiral pathogenicity. Fig. 4A shows the increased crawling fraction of the virulent cluster, whereas >75% cells remained in the swimming state in the non-infection cluster. This is consistent with the observation that the virulence pairs had a significantly larger $K_{A\text{-}C}$ in comparison with asymptomatic and non-pathogenic pairs ($P < 0.05$); $K_{S\text{-}A}$ did not seem to correlate to virulence (Fig. 4B). These thermodynamic parameters suggest that the biased transition from adhesion to crawling would be responsible for the virulence of *Leptospira*. Although the crawling speed could not be correlated to pathogenicity (Fig. 4C), a detailed analysis of the crawling patterns revealed that long-distance directive crawling was a crucial virulence factor (Fig. 4D). The crawling motility of *Leptospira* is caused by the attachment of the spirochete cell body to surfaces via adhesive cell surface components[20,24]. 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[20]. LPS, the molecular basis for the identification of the different *Leptospira* serovars[2,9], is thought to be a crucial adhesin important for this crawling motion[20]. 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. 4E). The biased transition from the adhesion to the crawling state and the long-distance, persistent crawling allows for the leptospires to explore the host's cell surfaces, increasing the probability of encountering routes for invasion through their intracellular tight junctions (Fig. 4E, left). In contrast, the weakly attached leptospires can detach easily and be swept by external forces, such as intermittent urination (Fig. 4E, right).

151 Adherence of microbes is generally important in the early stage of infection. For example,
152 the enteropathogenic *Escherichia coli* (EPEC) uses H2 or H6 flagella for adhesion[25], and
153 host-dependent pathogenicity of the EPEC strain RDEC-1 infection is associated to the
154 adherence affinity of the bacteria to the host intestinal epithelium[26]. Our results showed the
155 importance of adhesion to and persistent crawling on the host tissue for disease severity of
156 *Leptospira* infection, and concurrently highlighted the close relationship of such surface
157 dynamics of the spirochetal pathogen to their host-dependent pathogenicity. These findings
158 could lead to the development of antimicrobials for leptospirosis targeting the pathogens'
159 adhesion and crawling motility and provides a significant step forward to confront other
160 host-dependent bacterial infections. Meanwhile, microarray analyses have revealed that
161 regulation of gene expression in *Leptospira* is affected by its interaction with host cells[27,28].
162 Although host immune responses against *Leptospira* infection remain mostly unknown,
163 pathogenic leptospires interfere with the complement system through the degradation of
164 complement proteins[29] and inhibition of coagulation via binding to thrombin[30]. Thus, in
165 addition to bacterial dynamics, the molecular and immunological understanding of the
166 host-pathogen association will realize the establishment of more effective prevention
167 strategy.
168
169 **Methods**
170 ***Leptospira* strains and growth conditions.** Pathogenic serovars of *L. interrogans*
171 serovar Icterohaemorrhagiae (strain WFA135), an isolate from *Rattus norvegicus* in Tokyo,
172 Japan, serovar Manilae (strain UP-MMC-NIID)[31,32] and a saprophytic *L. biflexa* serovar
173 Patoc (strain Patoc I) were used in this study. The serovar of WFA135 was determined by
174 multiple loci variable number of tandem repeats analysis (MLVA) and DNA sequencing of the
175 *lic12008* gene[32,33]. Bacteria were cultured in enriched
176 Ellinghausen-McCullough-Johnson-Harris (EMJH) liquid medium (BD Difco, NJ, USA)
177 containing 25 μg/mL spectinomycin at 30 °C for 2 (*L. biflexa*) or 4 (*L. interrogans*) days until
178 the stationary phase. To track *Leptospira* cells when in co-culture with mammalian kidney
179 cells, a green fluorescent protein (GFP) was constitutively expressed in each strain (Fig. 1D;

Supplementary Movie 1).

Mammalian cells and media. Mammalian kidney epithelial cell lines used included MDCK-NBL2 (dog), NRK-52E (rat), Vero (monkey), MDBK-NBL1 (cow), TCMK-1 (mouse), and HK-2 (human). MDCK, Vero, TCMK, and MDBK cells were maintained in Eagle's minimum essential media (MEM) (Sigma-Aldrich, Darmstadt, Germany) containing 10% fetal bovine serum or 10% horse serum (Nacalai Tesque, Kyoto, Japan) at 37 °C and 5% CO_2. NRK cells were maintained in Dulbecco's modified Eagle's media (DMEM) (Thermo Fisher Scientific, MA, USA) with 4 mM L-glutamine, adjusted to contain 1.5 g/L sodium bicarbonate and 4.5 g/L glucose, and with 5% bovine calf serum (Nacalai Tesque) at 37 °C and 5% CO_2. HK-2 cells were maintained in keratinocyte serum-free (KSF) media with 0.05 mg/mL bovine pituitary extract and 5 ng/mL human recombinant epidermal growth factor (Gibco - Thermo Fisher Scientific, MA, USA). All culture conditions contained a 5% antibiotic / antimycotic mixed solution (Nacalai Tesque). Cells were treated with a 0.1% trypsin - EDTA solution (Nacalai Tesque) to dislodge the cells during each passage process.

GFP expression in *L. interrogans* and *L. biflexa.* For the construction of a replicable plasmid in *L. interrogans*, the *rep-parB-parA* region of the *L. interrogans* serovar Canicola strain Gui44[34] plasmid pGui1 was amplified from a *L. interrogans* serogroup Canicola isolate, and the amplified product was cloned into the PCR-generated pCjSpLe94[35] by NEBuilder HiFi DNA Assembly cloning (New England BioLabs), generating pNKLiG1. The *flgC* promoter region and *gfp* were amplified from pCjSpLe94 and pAcGFP1 (Clontech), respectively, and the amplified products were cloned into the *Sal*I-digested pNKLiG1 for *L. interrogans* or the *Sal*I-digested pCjSpLe94 for *L. biflexa*. The plasmids were transformed into strains WFA135, UP-MMC-NIID or Patoc I by conjugation with *E. coli* β2163 harboring the plasmid[36]. Primer sequences used in this study are listed in Supplementary Table 1. Expression of GFP did not affect motility in the *Leptospira* serovars (Supplementary Fig. 3).

Preparation of kidney cells and *Leptospira* cells in a chamber slide. Kidney cells were

209 harvested with 0.1% trypsin and 0.02% EDTA in a balanced salt solution (Nacalai Tesque)
210 and plated onto a chamber slide (Iwaki, Tokyo, Japan) using their corresponding media
211 without antibiotics. The slides were incubated for 48 h until a monolayer was formed and
212 washed twice with media to remove non-adherent cells. The cells were incubated for a
213 further 2 h at 37 °C and 5% CO_2. Approximately 500 ml of stationary phase *Leptospira* cells
214 were harvested by centrifugation at 1,000 *g* for 10 min at room temperature, washed twice in
215 PBS, then resuspended in the corresponding kidney cell culture media without antibiotics at
216 37 °C to a concentration of 10^7 cells / ml. These suspensions (1 ml) were then added into the
217 corresponding chamber slides containing the kidney cell layer, and the chamber slides were
218 incubated at 37 °C for 1 h.
219
220 **Microscopy observation and adhesion-crawling assay.** The movement behaviors,
221 swimming, adhesion and crawling of the *Leptospira* cells on the kidney cells were observed
222 using a dark-field microscope (BX53, Splan 40×, NA 0.75, Olympus, Tokyo, Japan) with an
223 epi-fluorescent system (U-FBNA narrow filter, Olympus) and recorded by a CCD-camera
224 (WAT- 910HX, Watec Co., Yamagata, Japan) at 30 frames per second. *Leptospira* cells were
225 tracked using an ImageJ (NIH, MD, USA)-based tracking system and the motion parameters
226 such as motile fraction, velocity and MSD were analyzed using Excel-based VBA (Microsoft,
227 WA, USA). The two-dimensional MSD of individual leptospiral cells during a period Δt was
228 calculated by the following equation: $MSD(\Delta t) = \langle (x_{i+\Delta t} - x_i)^2 + (y_{i+\Delta t} - y)^2 \rangle$, where ($x_i$, y_i)
229 is the bacterial position at *I* (see also Supplementary Fig. 1).
230
231 **Statistical analysis.** All experiments were performed in triplicate. Statistical differences
232 between data were evaluated using a Student's *t*-test. The data clustering was performed
233 independently for each experiment using the k-means method in OriginPro. The clustering
234 method grouped the data population into a specified number of clusters referring to the
235 Euclidian distance of each data point from the centroid of the cluster, calculated at every
236 clustering process, and reclassifying the data point to the nearest cluster.
237
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Competing interests The authors declare that they have no competing interests.

371 **Table 1. Host-dependent pathogenicity of *L. interrogans* serovars Manilae and**
372 **Icterohaemorrhagiae**

Serovar	Host	Major findings of infection (kidney)	References
Manilae	Rat	Persist in the kidneys causing either slight or no harm (maintenance hosts)	Faine et al. (1999), Villanueva et al. (2010), Villanueva et al. (2016)
	Dog	Unknown	NA
	Monkey	Unknown	NA
	Mouse	Pyelonephritis, chronic infection	Gomes-Solecki et al. (2017), Ratet et al. (2014), Fanton d'Andon et al. (2014)
	Cow	Asymptomatic carrier	Villanueva et al. (2016), Yanagihara et al. (2007)
	Human	Oliguria and/or proteinuria, hematuria, renal failure	Yanagihara et al. (2007), Masuzawa et al. (2001)
Icterohaem-orrhagia	Rat	Persist in the kidneys causing either slight or no harm (maintenance hosts)	WHO (2003), Faine et al. (1999), Koizumi et al. (2009)
	Dog	Dehydration, pyelonephritis, hematuria	Geisen et al.(2007), Rentko et al. (1992), Adin et al. (2000)
	Monkey	Hemorrhagic syndrome, renomegaly	Asai et al. (1991), Perolat et al. (1992), Szonyi et al. (2011)
	Mouse	Hemorrhage, pyelonephritis	Pereira et al. (1998), Gomes-Solecki et al. (2017)
	Cow	Hemoglobinuria, anemia, abortion, chronic carrier	Hassig et al. (1998), Guerra. (2009)
	Human	Oliguria and/or proteinuria, hematuria, renal failure	Levett (2001), Guerra. (2009), Faine (1999)

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374 Faine et al. (1999)[37]; Villanueva et al. (2016)[38]; Villanueva et al. (2010)[39]; Gomes-Solecki et
375 al. (2017)[40]; Ratet et al. (2014)[41]; Fanton d'Andon et al. (2014)[42]; Yanagihara et al. (2007)[43];
376 Masuzawa et al. (2001)[44]; World Health Organization (2003)[45]; Koizumi et al. (2009)[46];
377 Geisen et al. (2007)[47]; Rentko et al. (1992)[48]; Adin et al. (2000)[49]; Asai et al. (1991)[50]; Perolat
378 et al. (1992)[51]; Szonyi et al. (2011)[52]; Pereira et al. (1998)[53]; Hässig et al. (1998)[54]; Guerra
379 (2009)[55]; Levett (2001)[5].
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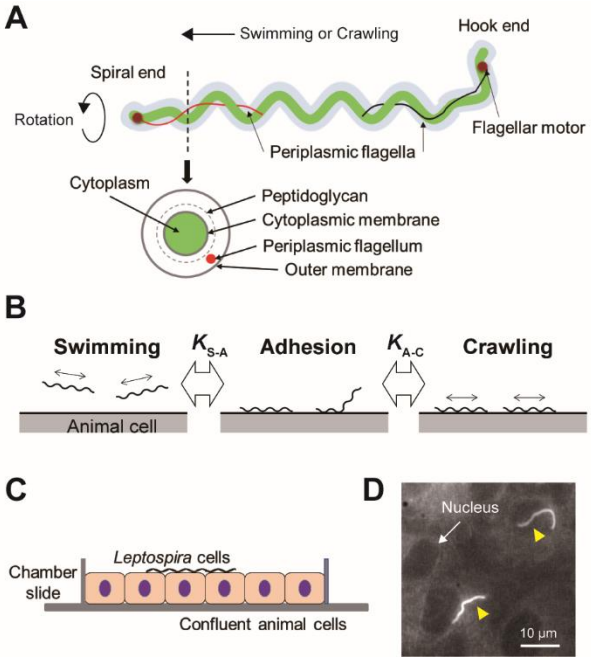

Fig. 1. Working model and schematic representation of the experimental setup. (A) Schematic diagram of the *Leptospira* cell structure. (B) A three-state kinetic model assuming a transition between "swimming" (floating above cell layers without physical contact to the cells) and "adhesion" (attachment to the cell layer without migration), and between "adhesion" and "crawling" (attachment to the cell layer and movement over surfaces), with $K_{S\text{-}A}$ and $K_{A\text{-}C}$ represent the equilibrium constants of each transition, respectively. (C) 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. Three *Leptospira* strains (the pathogenic *L. interrogans* serovars Icterohaemorrhagiae and Manilae, and the saprophytic *L. biflexa* serovar Patoc) and kidney epithelial cell lines from six species (MDCK-NBL2, dog; NRK-52E, rat; Vero, monkey; MDBK-NBL1, cow; TCMK-1, mouse; HK-2, human) were used in the experimental model. (D) 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 (Supplementary Movie 1).

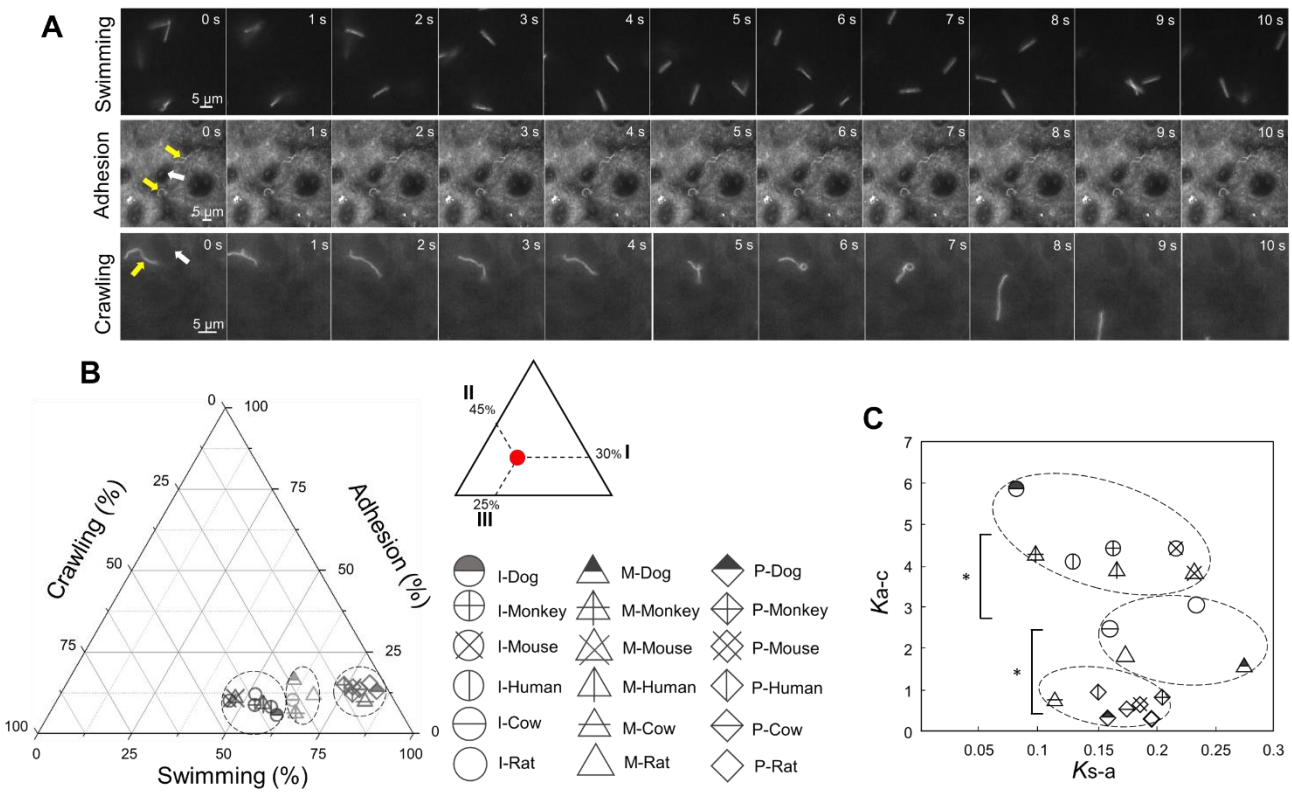

Fig. 2. Steady-state motility and adhesion of *Leptospira* cells on kidney cells. (A) Image sequences of swimming (top), adhered (middle), and crawling (bottom) leptospiral cells. Yellow and white arrows indicate *Leptospira* cells and kidney cells, respectively. No kidney cells were observed due to out of focus during measurement of *Leptospira* cells swimming in liquid media (**Fig. 1B**). (B) 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. Dashed circles indicate the results of the k-means clustering. (C) Pairwise plot of the equilibrium constants $K_{S\text{-}A}$ and $K_{A\text{-}C}$, calculated from the data shown in **B**. Dashed circles are the results of k-means clustering performed independently of **B**. The statistical analysis used the Student's *t*-test (* $P < 0.05$).

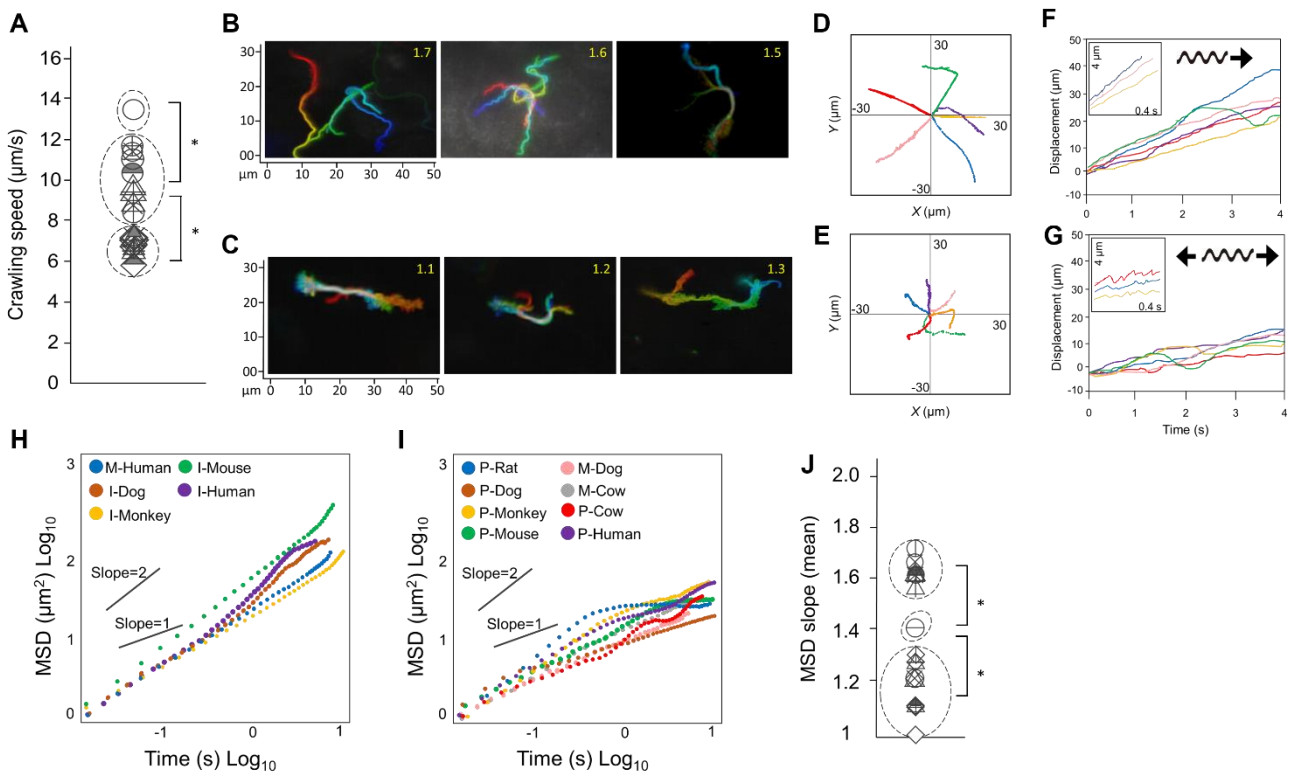

Fig. 3. Analysis of the crawling movement of *Leptospira* on kidney cell layers. (A) Average crawling speeds determined for each host-serovar pair. Examples of persistent crawling (B) and diffusive crawling (C) of *Leptospira* cells obtained by single-cell tracking in 10 s. Colors indicate time courses in the order of red orange yellow green blue, and indigo. (D-E) Examples of crawling trajectories of leptospires on the kidney cell surfaces. (F-G) Time course of the displacement obtained from **D** and **E**; inset, expanded view of cell displacements and schematics of motion patterns. **B**, **D**, and **F** show the long-distance migration represented by directive crawling; **C**, **E**, and **G** show the limited migration due to frequent reversal. (H-I) Evaluation of the directivity of individual leptospiral cell movements by MSD vs time plots. MSD plots with slopes ~ 2 indicate persistently directive crawling (**H**), whereas plots with slopes ~ 1 indicate a non-directional movement (**I**), 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 **F** and **G**. In **B** and **C**, the values of MSD slopes of each cell are shown in yellow numbers. (J) Average values of MSD slopes as determined from the MSD analysis. Refer to legend on **Fig. 2B** for symbols shown in **A** and **J**. Each data point was obtained from at least 90 leptospiral cells. Gray circles in **A** and **J** represent the results of the k-means clustering. Statistical analysis used the Student's *t*-test, (* $P < 0.05$).

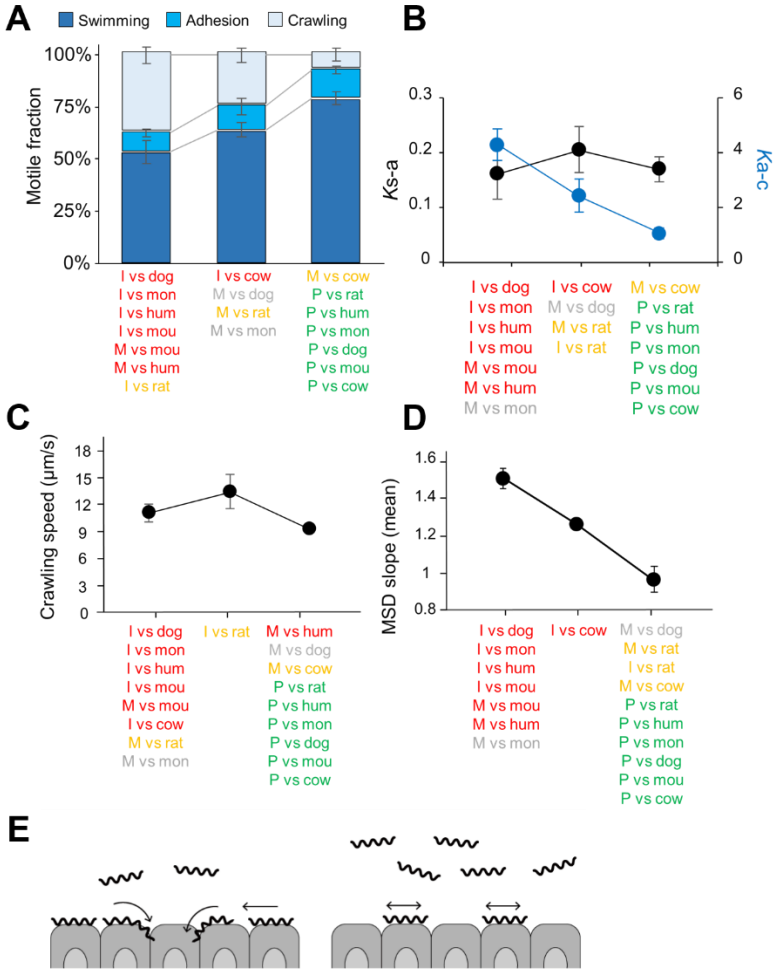

Fig. 4. Correlation of adhesion, motility and pathogenicity. According to the statistical classification in **Figs 2-3**, host-serovar pairs are listed below each figure. Based on a literature search summarized in **Table 1**, the examined pairs are indicated in red (with registered symptoms), yellow (asymptomatic or natural reservoir), green (non-pathogenic *Leptospira*), or grey (unknown infection outcomes). The correlations of (A) the cell fractions in each state, (B) the thermodynamic parameters, (C) the crawling speed, and (D) the MSD slopes (i.e., the directivity, or persistency of crawling) to the reported clinical consequences (**Table 1**) are shown. **A**-**D** show the mean values and standard deviations (error bars) calculated for each cluster using the values shown in **Fig. 2** and **Fig. 3**. (E) 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.