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学位論文　（要約）

Development of an enzyme-linked immunosorbent assay system based on recombinant leptospiral outer membrane protein LipL32 expressed by *Escherichia coli* and *Pichia pastoris* for *Leptospira* infection in rodents.

（大腸菌およびメタノール資化酵母により発現させた
組換えレプトスピラ外膜蛋白 LipL32 を用いた
げっ歯類レプトスピラ感染症診断 ELISA 法の開発）

２０１５年　６月

北　海　道　大　学

塩川　愛絵

Kanae SHIOKAWA

Introduction

Leptospirosis is a bacterial zoonotic disease caused by spirochetes of the genus *Leptospira*. *Leptospira* spp. (Figure 1, A) are classified into pathogenic and saphrophytic species. Pathogenic leptospires consist of more than 260 serovars. Serovars are defined by cross-absorption and agglutination tests with homologous and heterologous immune sera[1]. The large variety of pathogenic serovars makes it difficult to develop vaccines because of the serovar specificity of immunoreactions[1].

A wide range of susceptible animals are potential reservoirs of leptospires including companion animals, livestock, wildlife and peridomestic animals such as rats and mice. Serovars are typically associated with a particular species of the natural host, although there are many exceptions as one serovar may be carried by different hosts and one animal species may act as a host to several serovars. Rodents play an important role as a source of human infection as they have persistent asymptomatic infection with leptospires and shed them in the environment throughout their life[2-6] (Figure 1, B). Leptospirosis is incidental and results from direct or indirect exposure to the urine of infected animals.

Emerging outbreaks of leptospirosis have been reported after natural disasters and severe weather such as a typhoon, hurricane and heavy rainfall in tropical and subtropical regions[7,8]. Leptospirosis cases have been reported from all over the world except the north and south polar regions, and large numbers of cases occur in Central and South Americas, Western Pacific and South East Asia. The annual incidence of severe leptospirosis cases was estimated by International Leptospirosis Society surveys to be 350,000 to 500,000. The annual incidences in endemic and epidemic areas of human leptospirosis were reported by the Leptospirosis Burden Epidemiology Reference Group established by WHO to be 5 and 14 cases per 100,000 population respectively. Reported cases and minor outbreaks after various outdoor activities such as swimming, hiking, and rafting have also been increasing in recent years in endemic areas of leptospirosis[9,10]. Therefore, it is essential to obtain epidemiological information on reservoir animals of *Leptospira* spp. and *Leptospira*-contaminated environments from a preventive public health perspective [3,5].

Taxonomy of *Leptospira*

The first isolation of leptospires was reported in 1914 by Wolbach and Binger[11]. Historically, isolates were differentiated as strains by agglutination tests. Antigenically distinct strains were assigned the status of species. However, the difficulty in differentiation from existing species became a concern with the proliferation of new species. In 1954, Wolff and Broom proposed the use of the term serotype strain, now called serovar, for the basic taxonomic unit of serological classification instead of ascribing species names for serologically distinct strains.

In 1957, the genus *Leptospira* was divided into two species, *Leptospira interrogans* sensu lato and *Leptospira biflexa* sensu lato, in the 7th edition of Bergey's Manual. At that time, the two species were differentiated by phonotypic features depending on the culture condition. *L. biflexa* can grow at 13 ℃ in the presence of 8-azaguanine (225 ug/ml), whereas *L. interrogans* requires a culture temperature of 28 to 30 ℃ for isolation and was lethal in the presence of 8-azaguanine [12-14]. The species *L. interrogans* includes more than 260 serovars, all pathogenic serovars and some saprophytic serovars. On the other hand, the species *L. biflexa* has only saprophytic serovars.

A genotypic classification has been established as a number of so-called genomospecies include all serovars of both *Leptospira interrogans* sensu lato and *Leptospira biflexa* sensu lato. Various molecular typing methods such as digestion of chromosomal DNA with restriction endonucleases (REA), restriction fragment length polymorphism (RFLP), ribotyping, pulsed-field gel electrophoresis (PFGE), and a PCR-based method have been used for genotyping. However, the genomospecies by genotypic classification do not correspond to the phenotypic and serological classification, for which there are two species and serovars[15,16]. This inconsistency between the phenotypic-serological classification and the genotypic classification has caused confusion among clinical microbiologists, clinicians and epidemiologists who have been using the phenotypic-serological classification. Despite the difficulties in integrating classifications of leptospires isolates, much interest has been shown in genomospecies analysis for further identification and subtyping of leptospires under the Leptospira Genomic and Human Health project [17,18].

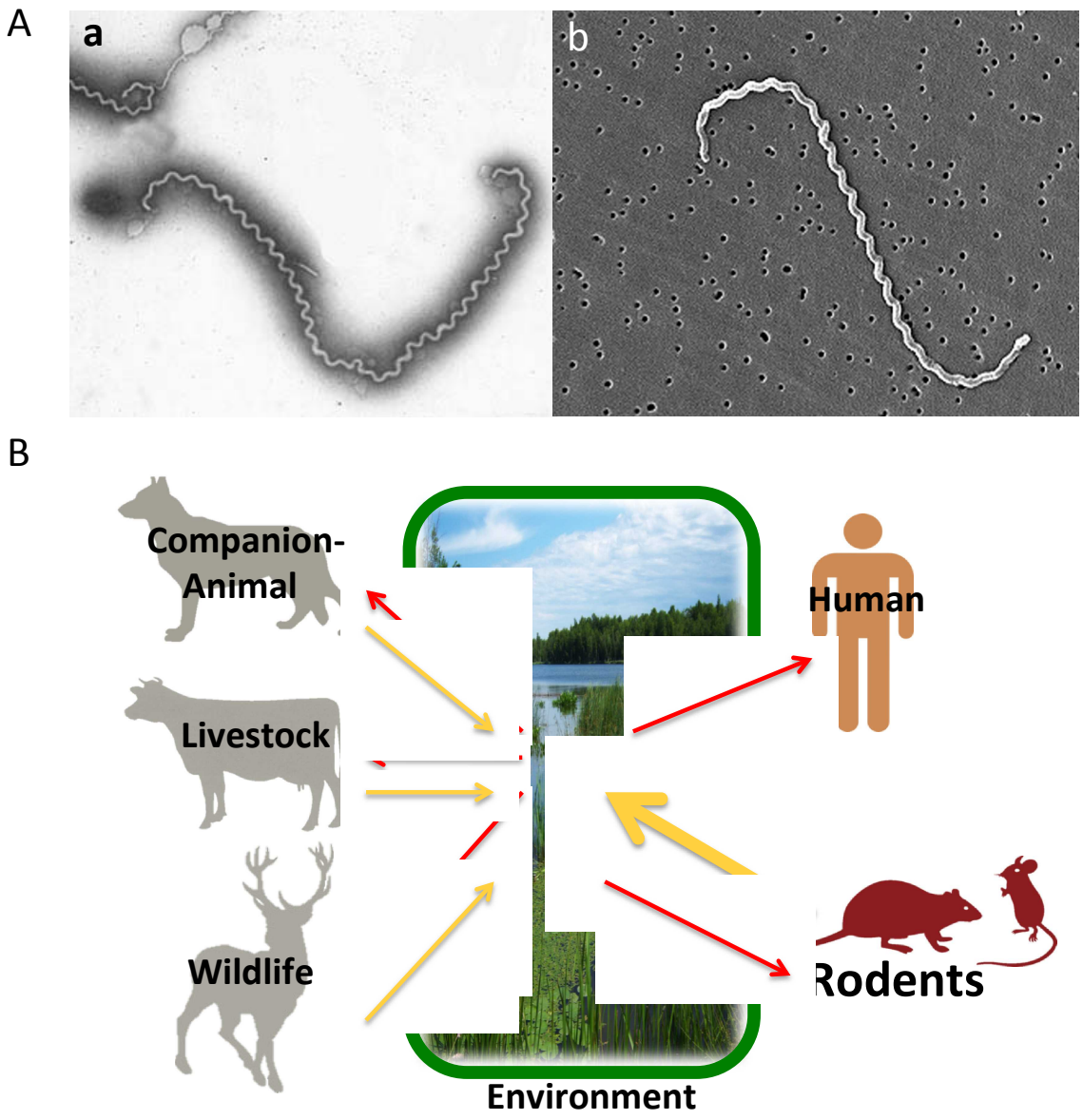


Figure 1. Epidemiology of leptospirosis

(A) (a) Scanning electron micrograph of *Leptospira* spp. shows the corkscrew appearance at either end of the bacterium. [Source: Center for Veterinary Education, the University of Sydney (http://www.vetbook.org/wiki/dog/index.php/Leptospira_spp)]
(b) Scanning electron micrograph of *L. interrogans* serovar icterohaemorrhagiae strain RGA on a 0.2-um pore filter membrane. [Source: modified from Levett 2001[1], Weyant, R. et.al 1990]

(B) Cycle of leptospiral infection
Susceptible animals excrete pathogenic *Leptospira* spp. in their urine and play an important role as reservoirs for their transmission. Pathogenic *Leptospira* spp. are maintained in sylvatic and domestic environments among rodent species. Rodents are considered to be chronic and asymptomatic carriers. *Leptospira* infection of livestock and of domestic and wildlife animals causes a wide range of disease manifestations and carrier states. Human leptospirosis occurs by direct or indirect contact with reservoir animals or by exposure to environments such as water or soil that are contaminated with infected animal's urine. Humans are considered to be accidental hosts and would not be a reservoir for transmission without shedding sufficient numbers of leptospires.

Vaccines

Current leptospiral licensed vaccines are only whole cell-based killed vaccines. Bovine, porcine, canine and human vaccines are commercially available[19-22]. Human leptospiral vaccines have not been applied widely except in occupational infection risk management. Unfortunately, these current vaccines have high rates of adverse reactions due to the presence of lipopolysaccharide (LPS) and culture media. Moreover, these vaccines induce short-term immunity with an annual booster injection being required to maintain immunity over the protected level. The induced immunity is serovar-specific and only protects against the infections of *Leptospira* with homologous serovars or antigenically similar serovars. Thus, it is also critically important to isolate and identify locally prevalent serovars for preparing efficient inactivated vaccines.

Despite efforts having been made in immunization, some current vaccines would not be effective for preventing infection and carriage of *Leptospira* but have shown to be effective for preventing severe symptoms as a homologous serovar transmission case from immunized dogs to humans had been reported[23]. Attenuated vaccines and component vaccines using leptospiral lipopolysaccharide and recombinant proteins are currently being studied.

Symptoms and treatment

Leptospirosis patients develop a wide range of symptoms from subclinical infection to high fever, headache, muscular pain, abdominal pain, intense jaundice, bleeding, renal and pulmonary dysfunctions, and neurologic alterations[5]. Severe cases are also known as Weil's disease or leptospirosis pulmonary haemorrhage syndrome (LPHS)[24,25], and fatality rates of those cases are >10% and >74%, respectively[26] (Figures 2 and 3). Correct diagnosis and appropriate treatment at an early stage are important for a good prognosis. If early treatment is not provided, the disease progresses to severe multisystem manifestations such as hepatic dysfunction and jaundice, acute renal failure, pulmonary haemorrhage syndrome, myocarditis and meningoencephalitis during the late immune phase. The severity level of leptospirosis symptoms is not predictable from an infected serovar type. Livestock leptospirosis causes abortion, hepatitis, nephritis, stillbirth and placentitis, equine leptospirosis causes uveitis, and canine leptospirosis is known to have a tendency for being acute severe leptospirosis and LPHS (Figures 2 and 3).

There is a risk of misdiagnosis in human leptospirosis since symptoms of leptospirosis are similar to symptoms of other diseases such as malaria, viral hepatitis, yellow fever, dengue, chikungunya fever, hantavirus infections, and bacterial and viral

meningitis, especially in areas where the diseases are co-circulating. Furthermore, infectious diseases such as leptospirosis, hantavirus infections and dengue are thought to play an important role in chronic kidney disease, which became epidemic in Central America[27-29]. The lack of appropriate treatment in the early stage of infection will lead to a long-term burden of diseases.

Antibiotics are effective for treatment of leptospirosis. Doxycycline is recommended for mild cases and β -lactams (e.g., penicillin and amoxicillin) are effective for the early stage of infections [30-32]. However, penicillin can cause a shock syndrome known as Jarisch-Herxheimer reaction by leptospiral exotoxins [33,34]. Active prophylaxis with doxycycline is recommended for people at high risk for infections [35].

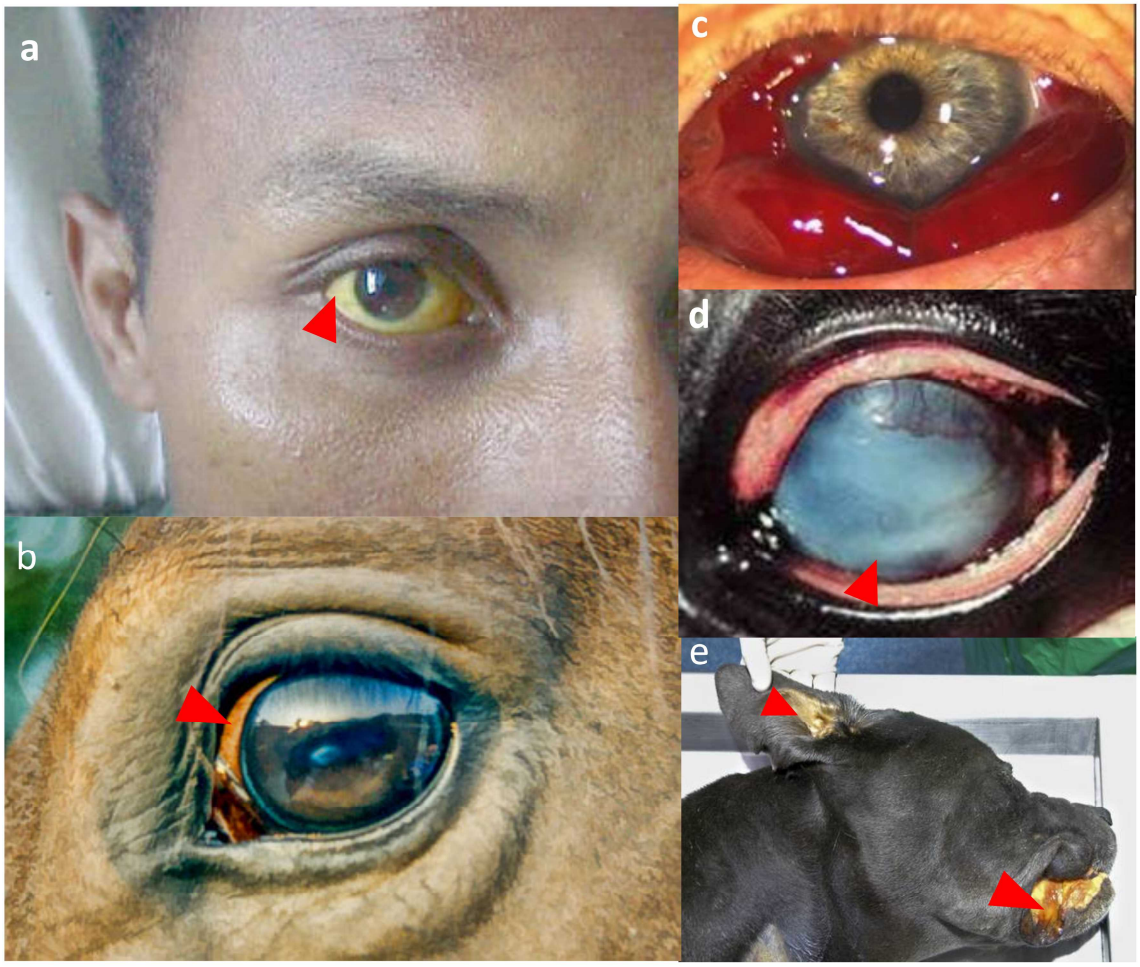
A



Figure 2. Symptoms of Leptospirosis

A: Human leptospirosis with purpura.
[Source: HSE professional.org (http://www.hseprofessional.org/leptospirosis/)]

B



B:

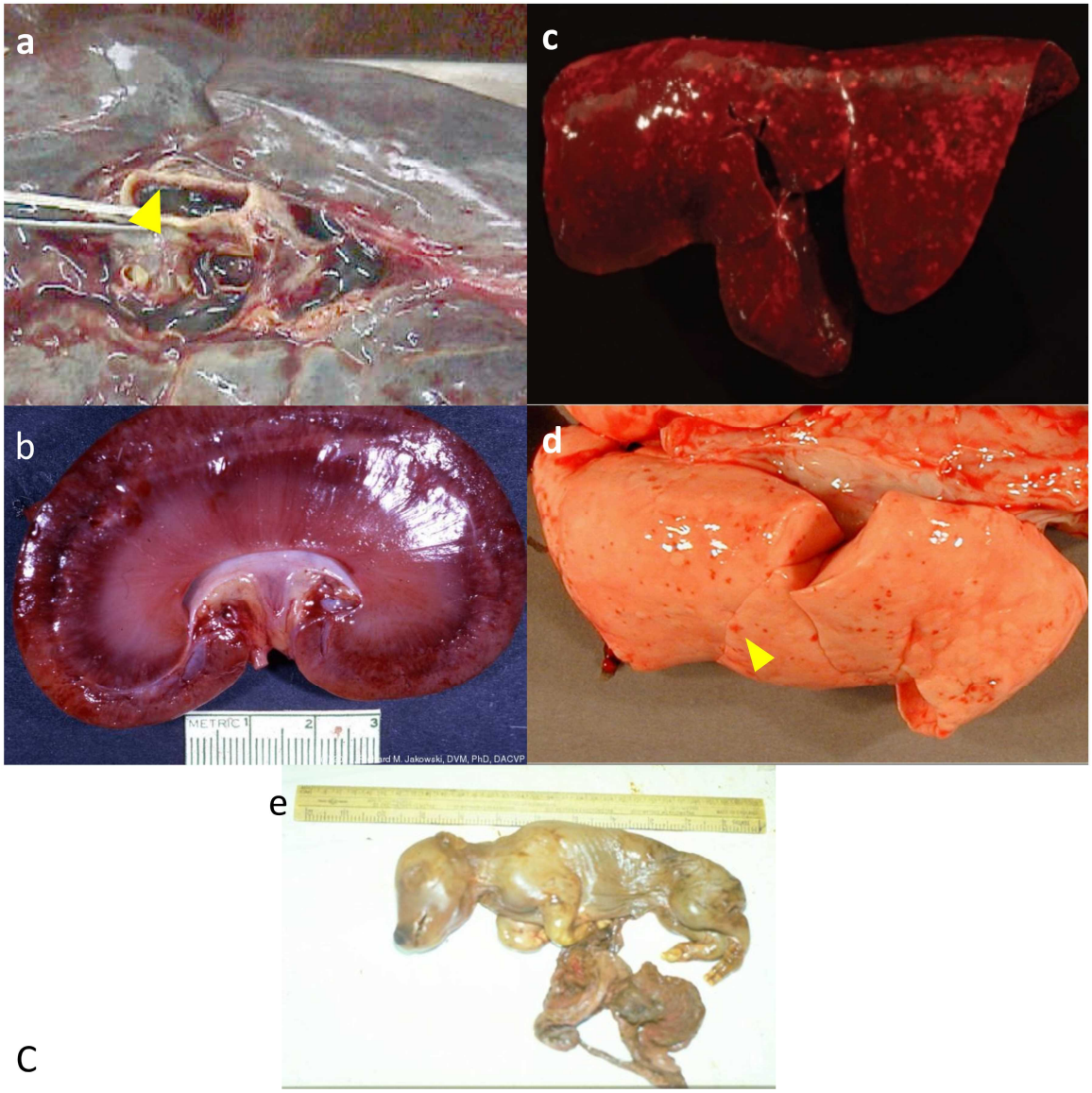
(a) Human leptospirosis with jaundice (arrowhead). [Source: modified from Michael Lowe, The Fiji School of Medicine and the Fiji School of Nursing (http://www.fsm.ac.fj/Medicine%20Website/HADIF%20website/)]

(b) Equine leptospirosis with uveitis and jaundice (arrowhead). [Source: modified from Vet next (http://www.vetnext.com/search.php?s=aandoening&id=73059336071%20414)]

(c) Human leptospirosis with uveitis. [Source: the American Academy of Optometry (http://www.aaopt.org/)]

(d) Equine leptospirosis with uveitis (Moon Blindness). Inflammatory cells in the anterior chamber form a hypopyon (arrowhead). [Source: modified from Vet next (http://www.vetnext.com/search.php?s=aandoening&id=73059336071%20414)]

(e) Canine letospirosis with jaundice (arrowheads). [Source: modified from MSD animal health (http://www.msd-animal-health.ie/diseases/pets/infectious_diseases/leptospirosis/Clinicalsigns.aspx)]



C

C:

(a) Canine leptospirosis. Pulmonary haemorrhage and intrabronchial blood pooling (arrowhead) . [Source: modified from Michael Lowe, The Fiji School of Medicine and the Fiji School of Nursing (http://www.fsm.ac.fj/Medicine%20Website/HADIF%20website/)]

(b) Canine leptospirosis. Pulmonary haemorrhage syndrome. [Source: Center for Veterinary Education, the University of Sydney (http://www.vetbook.org/wiki/dog/index.php/Leptospira_spp)]

(c) Canine acute leptospirosis with kidney cortical hemorrhage. [Source: modified from Tufts OpenCourseWare, Tufts University (http://ocw.tufts.edu/Content/72/imagegallery/1362318/1368968/1376627)]

(d) Canine leptospirosis with pulmonary petechiations (arrowhead). [Source: modified from Center for Veterinary Education, the University of Sydney (http://www.vetbook.org/wiki/dog/index.php/Leptospira_spp)]

(e) Porcine leptospirosis. Late mummification of the fetus. [Source: National Animal Disease Information Service, United Kingdom (http://www.nadis.org.uk/bulletins/leptospirosis-in-pigs.aspx)]

Diagnosis

For accurate diagnosis, it is important to use an appropriate diagnosis system with specimens that were collected at an appropriate time and from an appropriate biological sample (Figure 3).

Isolation of leptospires and the cross agglutinin adsorption test (CAAT) have been one of the leptospirosis diagnosis standards, but isolation culture requires a few weeks to one month with a low success rate.

Most cases of human leptospirosis are diagnosed by serology. The microscopic agglutination test (MAT), to identify the serovar-specific antibody in sera, has been considered the golden standard for serodiagnosis (Figure 4). However, the MAT has a tendency to fail to detect an antibody against *Leptspira* in serum specimens that were collected during the acute infection phase, approximately 5 to 7 days after onset of disease, when the serum level of serovar-specific antibody is low[1]. Other disadvantages of the MAT are that it is time-consuming and formidable procedures, and a requirement to keep quality controlled a panel of reference serovars and each of those corresponding rabbit standard sera in biosecurity facilities. A panel of reference serovars consists of live cultures of leptospires and it is therefore difficult to conduct the MAT without proper biosecurity infrastructures[36]. Both isolation and the MAT are not suitable for clinical diagnosis, which requires promptness and simplicity. However, isolation and the MAT are still essential for an epidemiological study on the prevalence of leptospires in an area to provide basic information for vaccine production[5,37,38].

Rapid serological diagnostic systems have been developed using assays such as enzyme-linked immunosorbent assay (ELISA) and lateral flow assay (LFA), and some molecular diagnostic systems have been developed using PCR to detect genes of leptospires components such as *lipl32*, *flaB*, and *gyr B* or to amplify 16S ribosomal RNA and 23S ribosomal RNA [38-40]. The same techniques have generally been applied to reservoir animals for epizootiologic study and veterinary practice[41].

Recently, some recombinant leptospiral outer membrane proteins have been developed for serodiagnostic antigens, including LipL32, LipL21, LipL41, Omp1, LigA and LigB, which are convenient for quality control of culture systems compared to live leptospires cultures[42-45]. LipL32 is the most abundant lipoprotein expressed on the bacterial membrane, and it would thus be highly immunogenic and induce immunoreactions in the early stage of infection[46]. Additionally, LipL32 is highly conserved among pathogenic *Leptospira*[47] and is thought to exist only in pathogenic *Leptospira*. Therefore, LipL32 is a potential marker of pathogenic *Leptospira* for

leptospirosis diagnosis [48]. Applications of recombinant LipL32 for serodiagnosis have already been studied using canine, equine, bovine, and human sera[43,49-52].

Prevention and control of leptospirosis

Prevention and control of leptospirosis are based on local epidemiology, identifying circulating serovars and reservoirs in an area. Based on the epidemiological information, efficient measures including reductions of exposure, implementation of protective measures, immunization and chemoprophylaxis can be taken for reducing the risk of leptospirosis. Natural disaster control programs and pre-exposure chemoprophylaxis for outdoor activists and workers in leptospirosis-related occupations such as mining, cleaning sewers and farmers are carried out as strategies for prevention and control of leptospirosis. Vaccination is an extremely important strategy for prevention of leptospirosis in livestock and companion animals. Abortions caused by leptospirosis in livestock are calamitous for farmers[53], and leptospirosis in companion animals is a risk for human leptospirosis. Control of leptospirosis in animals will contribute to reductions in medical and veterinary medical costs, which is very important in developing countries.

Leptospirosis is also one of the poverty-related diseases that occur in conditions of inadequate sanitation without well-managed water supply and sewerage systems or good housing standards[54]. In an unsanitary living environment, there is a risk of exposure to rodents, which are common peridomestic animals[55]. Although leptospires infect a wide range of susceptible animals, rodents are the most important reservoirs that carry and excrete *Leptospira* over a long duration.

It should be noted that rodents are reservoir animals of various zoonotic agents including plague, leptospirosis, hantavirus infections, and hepatitis E virus infection [6,56]. Rodent-associated health risks are higher in an urban environment because an urban environment is an optimal habitat for rodents and there would be close and frequent contact between humans and rodents [57]. Progress in epidemiological research on rodent-borne diseases and identification of environmental risk factors for zoonotic diseases will increase public health awareness among healthcare and public health professionals that will reduce risks of misdiagnosis or underestimation of these diseases.

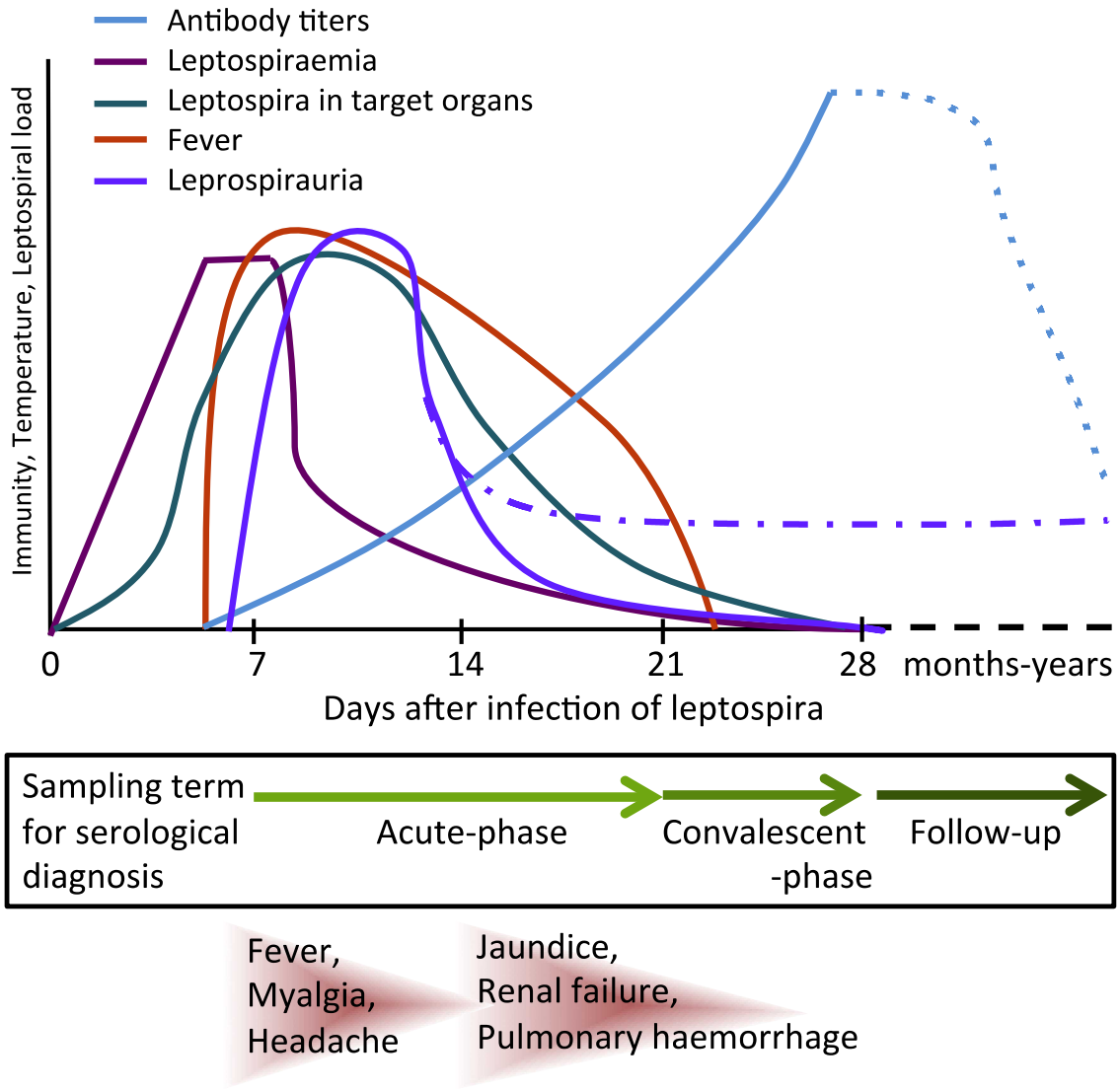


Figure 3. Leptospirosis kinetics

Leptospirosis causes fever, myalgia and headache during the early leptospiraemic phase after an incubation period of about 2 to 20 days. Leptospires persist for a long period in immunoprivileged sites such as renal tubules and the anterior chamber, causing prolonged leptospirauria (broken purple line), chronic kidney diseases and uveitis. Antibody titers usually decrease eventually, though antibody titers differ greatly between individuals and also depend on the levels of contamination by pathogens in the surrounding environment (broken blue line).

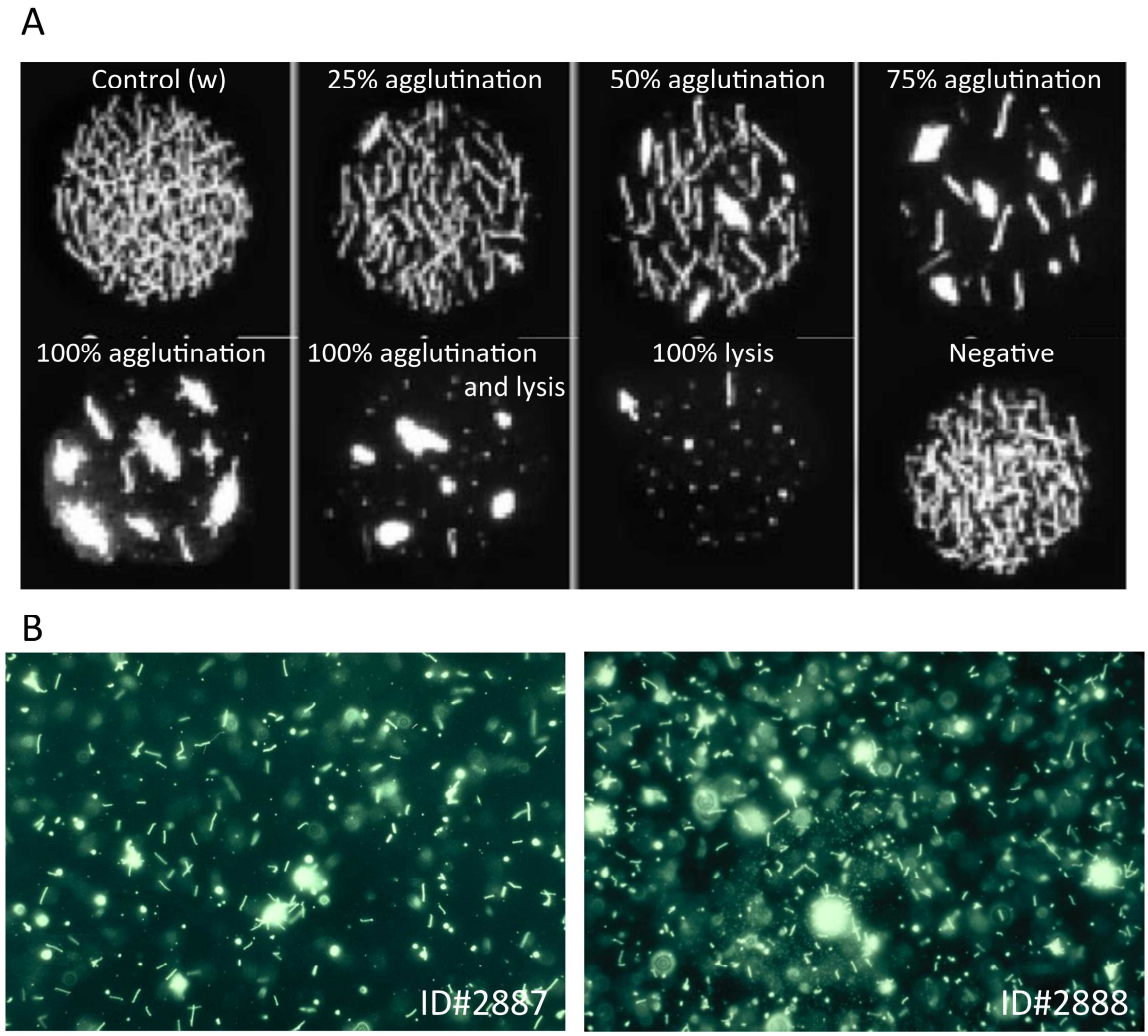


Figure 4. Microscopic agglutination test (MAT)

A: Reference of MAT results

Serum showing agglutination of at least 50% of the *Leptospira* is considered positive. It is important to keep in mind that antibody titers against local isolates are normally higher than titers against reference strains. The titer for the cutoff point is also determined carefully as a low titer such as 1/100 or 1/200 that would be appropriate only for populations in which the incidence of leptospirosis is low. More than 1/800 or higher titers should be considered as a determination of leptospirosis in endemic areas. Titers after the acute infection period are generally extremely high, such as 1/25600. [Source (in Spanish): modified from Manuel Céspedes Z, ARTÍCULO DE REVISIÓN, Leptospirosis: Enfermedad Zoonótica Emergente]

B: Leptospiral MAT with live antigen using darkfield microscopy

[Source: modified from the CDC and the Public Health Image Library, ID#2888, ID#2887 (http://phil.cdc.gov/phil/home.asp)]

Aim of the study

Peridomestic rodents are recognized as the main source of human leptospirosis worldwide. The presence of leptospires in rodent kidneys has recently been studied mainly by using PCR analysis[3]. However, application of a recombinant leptospiral antigen for rodent serological surveillance is limited.

In this study, recombinant LipL32 was applied to a serological screening test for rodent sera. First, the antigenic property of recombinant LipL32 was evaluated by competitive ELISA using monoclonal antibodies against LipL32 and sera of laboratory rats inoculated with *Leptospira interrogans*. The results indicated that the immunodominant area of LipL32 was located in the intermediate region. Secondly, the intermediate region including the immnodominant region was expressed by *Escherichia coli* and *Pichia pastoris.* The utility of recombinant LipL32 for ELISA screening with rat sera was evaluated.

Results and Discussion

LipL32 is abundantly expressed on the bacterial outer membrane and is present exclusively in pathogenic *Leptospira* serovars[46,48]. Therefore, LipL32 has been applied for serological diagnosis of *Leptospira* infection as a conserved antigen among pathogenic *Leptospira* serovars[51]. Despite the importance of rodents as a source of human leptospirosis, application of LipL32 for serodiagnosis of rodents has not been reported to the best of my knowledge. Hence, this study aimed to establish recombinant LipL32-based ELISA for serological diagnosis in field rodents. Firstly, wLipL32 was expressed in *E.coli* and used as an ELISA antigen. However, wLipL32 degraded easily and showed rapid reduction of antigenicity, probably due to the size of the protein (data not shown). Thus, tLipL32 including a major epitope region defined by MAbs was designed. Previous studies also demonstrated that the immunodominant part was mainly the central portion of Lipl32[58-60]. This competitive inhibition study using MAbs and laboratory rat sera also confirmed that the immunodominat region is located in the central part.

tLipL32 was expressed by *E.coli* and it was able to differentiate experimentally infected laboratory rat sera from control rat sera in ELISA, indicating that tLipL32 is applicable as a serodiagnostic antigen. Subsequently, tLipL32e was evaluated with field rat sera in ELISA. Unfortunately, high background reactions were observed in field rat sera that showed negative results in WB and PCR. A similar phenomenon occurred and was a struggle of serodiagnosis for Lyme borreliosis among patients with other bacterial infections, viral infections, and autoimmune diseases[61-64]. Fawceett *et al*. reported that number of false positive cases in cases of Lyme borreliosis could be reduced by the adsorption of test sera with components of *E.coli*[61]. A similar result using tLipL32e with *E.coli*-adsorbed sera was obtained in the present study. The high background reaction was attributed to a reaction to contaminants of the expression host *E.coli* in the recombinant antigen. The background reaction was not a problem for testing laboratory infected rat sera. These results support the theory that experimental *Leptospira* infection studies cannot replicate field conditions as discussed previously[65]. The results of experiments in this study suggest that wildlife animals potentially have a high antibody titer against multiple microorganisms, most likely *E. coli,* that vary depending on the environment. This is an important reminder when applying recombinant antigens expressed by *E.coli* to a serodiagnosis system. A comparison of results from multiple diagnosis approaches may be appropriate for research on prevalence in wildlife.

In this study, saccharomycetaceae *P. pastoris* was used as another expression host to reduce background reactions against *E. coli* components. It was confirmed that tLipL32p caused less background reactions in ELISA with field rat sera. This result additionally supports the theory that the high background reactions in tLipL32e-ELISA were reactions against components of *E.coli*.

Generally, the sensitivity and specificity of a novel diagnostic system are determined on the basis of diagnostic accuracy using true positive specimens and true negative specimens. However, definitions of true positive and true negative in wildlife are almost impossible. True positive and true negative field rat sera were tentatively defined on the basis of results from PCR and WB, and then distributions of ELISA OD values in the true positive and true negative groups were compared to determine the usefulness of truncated LipL32 for an ELISA antigen. tLipL32p-ELISA with untreated sera and tLipL32e-ELISA with adsorbed sera were able to discriminate OD distributions in positive and negative groups. On the other hand, tLipL32e-ELISA with untreated sera could not discriminate OD distributions. Based on the above understanding, tLipL32p is a valuable antigen for serological study in field rodents as it does not require sera adsorption by *E.coli*.

It is considerable rodents have a unique interaction with *Leptospira* to allow it in an immunological escape mechanism. Regarding the difference in that had been shedding period, it was reported that laboratory-raised wild Norway rat males had been injected with *L. interrogans* serovar icterohaemorrhaiae shed leptospires in urine 220 days after injection, whereas rats that had been injected with *L. interrogans* serovar grippotyphosa shed leptospires in urine 40 days after injection[66]. Laboratory environment and the route of infection doesn't represent the conditions of field natural infected animals though, it is noteworthy as rodents are recognized as influential reservoirs in the transmission of leptospirosis to humans [57,65]. *Rattus norvegicus* (Norway rat) and *Rattus rattus* (Rat), typical urban animals throughout the world, are also known as reservoirs for *L. interrogans*serovar icterohaemorrhagiae and *L. interrogans* serovar copenhageni [67]. The prevalence of *Leptospira* infection among rodents was shown to be dependent on population density and environmental conditions in addition the differences of individual sensitivity to leptospires and of harboring serovar[65]. *Leptospira* infection is considered to be a Susceptibles-Infectives-Susceptibles (SIS) model among deterministic compartmental models, because the vaccine effect is based on each serovar and there is the possibility of infection by other serovars. With the elimination of rodents in an area, another rodent population harboring novel serovars will come into the area. But also the endemic rodents population that have ever infected *Leptospira* will not be an

immunological barrier against spread of the disease in the area. Thus, preventive public health programs may be the best way to prevent leptospirosis, and epidemiological data for rodents in an area will provide important information to establish a public health strategy for leptospirosis.

Rodents are critical vectors of not only leptospirosis but also other zoonotic diseases such as plague, hantavirus infections, and hepatitis E infection[6,56]. Therefore, monitoring of the prevalence in rodents would provide valuable information for preventive medicine. However, detection of multiple pathogens may be burdensome due to the requirement of different specimens and types of test. On the other hand, serological research can provide information on multiple infections at the same time.

Genus-specific antibody detection ELISA using the non-pathogenic *Leptospira*, *L. biflexa* serovar Patoc is approved as an alternative serodiagnosis system for human leptospirosis[68]. However, 95% of the field rats were diagnosed by this system as being positive for *Leptospira* infection. This result suggests that either *L. biflexa* serovar Patoc dwells at a high density in the habitat of those rats or those rodents caused high background reactions in the ELISA. This genus-specific antibody detection ELISA may have the potential to cause overestimation of the prevalence of *Leptospira* infection in field rodents.

The recombinant LipL32-based ELISA used in this study has advantages for laboratories with few resources. The procedures for expressing a recombinant antigen are basically standardized and are safer than handling live leptospires. Recombinant protein-based ELISA also allows easier control of antigen quality and quantity than does the MAT using live leptospires. Moreover, diagnostic LipL32 antigen is promising for detecting only antibodies against pathogenic *Leptospira*. The aim of this study was to show the applicability of tLipL32p for ELISA using field rodent sera. Together with the results reported by Fawceett *et al*. that showed the effect of *E.coli*-adsorbed human sera in ELISA, the results for tLipL32p-ELISA in this study should lead to the application of tLipL32p for human leptospirosis serodiagnosis.

It has been reported that the renal leptospiral load in Norway rats showed unexpectedly high variations[66]. This means there is a risk to over-estimate the prevalence of *Leptospira* in rodents if only a single diagnosis system is used without knowing the pathological features of leptospirosis in rodents at that time. Therefore, I propose the use of both serological and molecular detection approaches, such as ELISA or WB and PCR, in parallel to provide more reliable results for research on the prevalence of field rat *Leptospira* infection.

A limitation of this study is that the study population was small. Further studies with larger numbers of field rat sera is needed to obtain a more accurate cutoff point in tLipL32-ELISA.

Conclusions

Recombinant LipL32-based ELISA was developed for serodiagnosis of *Leptospira* infection in rodents in this study. In conclusion, truncated LipL32 expressed by *P. pastoris* was shown to be the most valiant recombinant antigen for ELISA. Further study is needed to improve the efficacy of LipL32 and to determine appropriate combinations of conserved pathogenic *Leptospira* antigens for serodiagnosis in field rodents.

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