学位論文

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）

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Evaluation of truncated LipL32 expressed by *Escherichia coli* and *Pichia pastoris* for serodiagnosis of *Leptospira* infection in rodents
Comparative Immunology, Microbiology & Infectious Diseases, (under reviewing)

Parts of this research contents were presented in following scientific conferences.

1. **塩川 愛絵**、Chandika Gamage、小泉 信夫、迫田 義博、清水 健太、津田 祥美、吉松 組子、有川 二郎
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2. **塩川 愛絵**、Chandika Gamage、小泉 信夫、迫田 義博、清水 健太、津田 祥美、吉松 組子、有川 二郎
組換え抗原発現系の違いによる野生ラットレプトスピラ感染診断時のバックグラウンド反応軽減について
−大腸菌と酵母菌発現組換え病原性レプトスピラ共通抗原（LipL32 抗原）の比較−
第11回 北海道実験動物研究会学術集会、2014年7月26日、札幌、日本

3. Kanae Shiokawa, Chandika D Gamage, Nobuo Koizumi, Sanae Nishio, Yu Koarashi, Yoshimi Tsuda, Kenta Shimizu, Kumiko Yoshimatsu, Jiro Arikawa
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8th Scientific Meeting of International Leptospirosis Society 2013, Fukuoka, Japan, 8-11 October 2013

4. **塩川 愛絵**、吉松 組子、津田 祥美、清水 健太、有川 二郎
組換え抗原を利用したげっ歯類のレプトスピラ症迅速簡易診断法の開発
-Application of recombinant antigens for the antibody detection of the pathogenic leptospira in rodents sera-
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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].

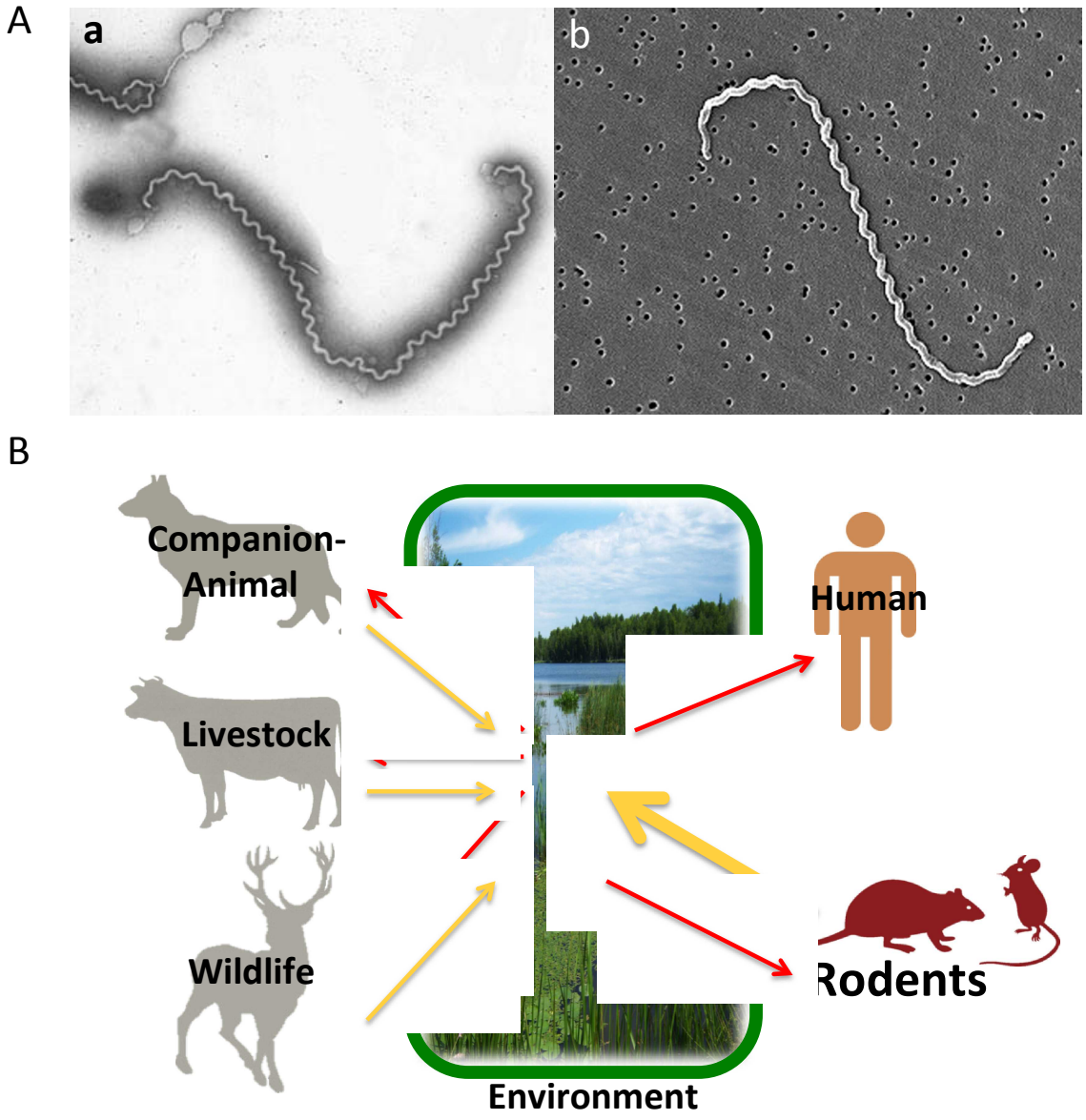


Figure 1. Epidemiology of leptospirosis

(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

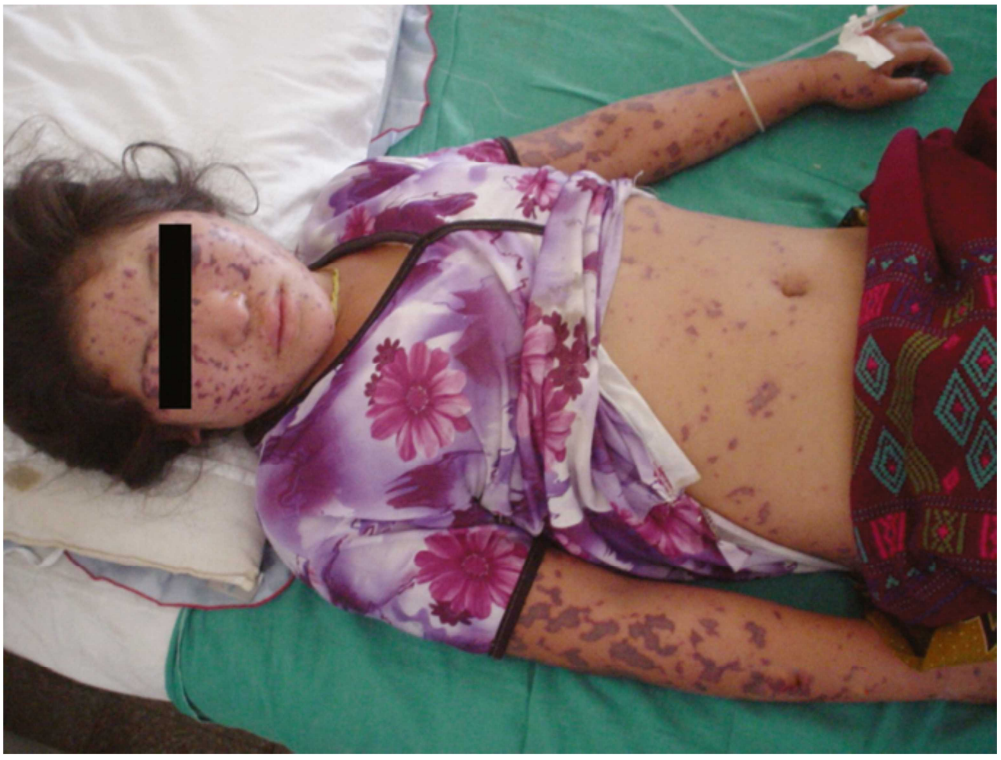
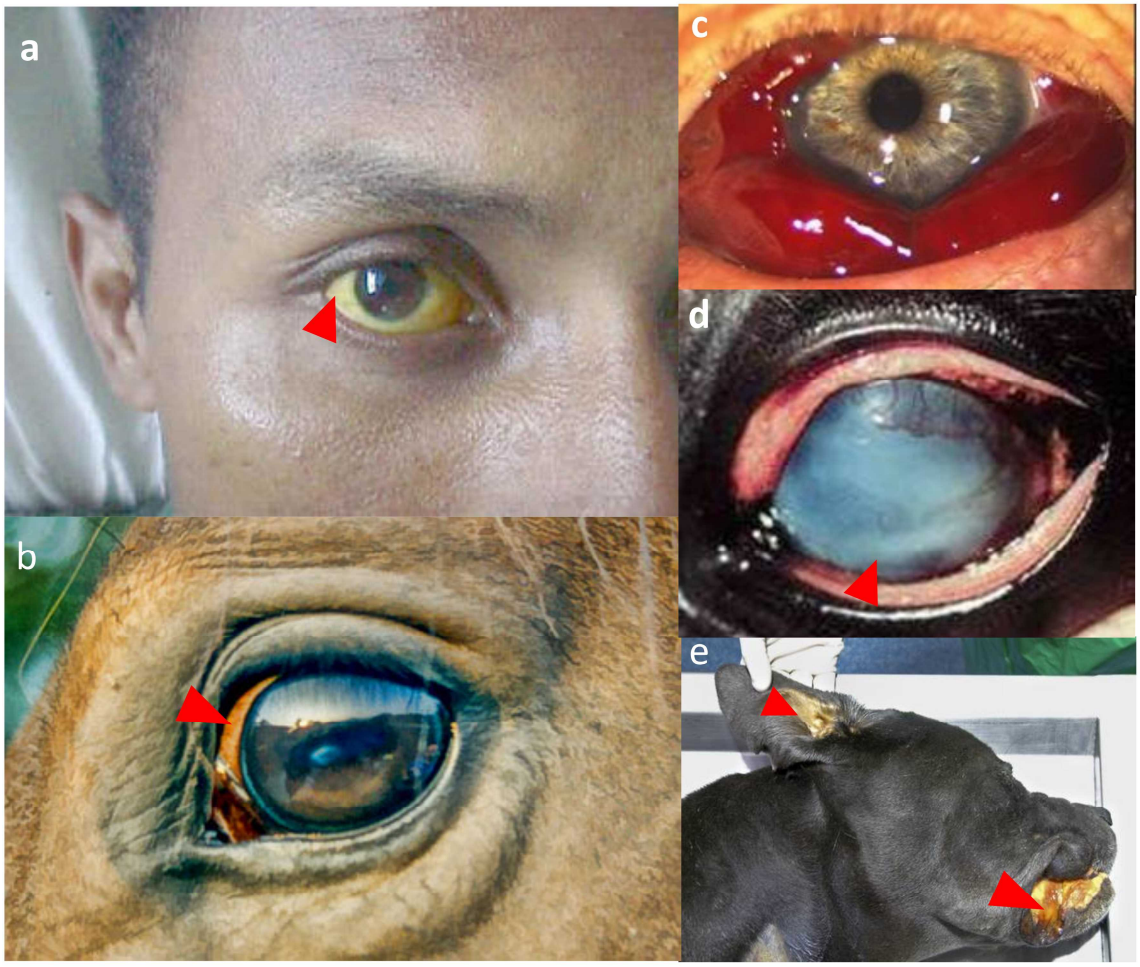


Figure 2. Symptoms of Leptospirosis

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

B



B:

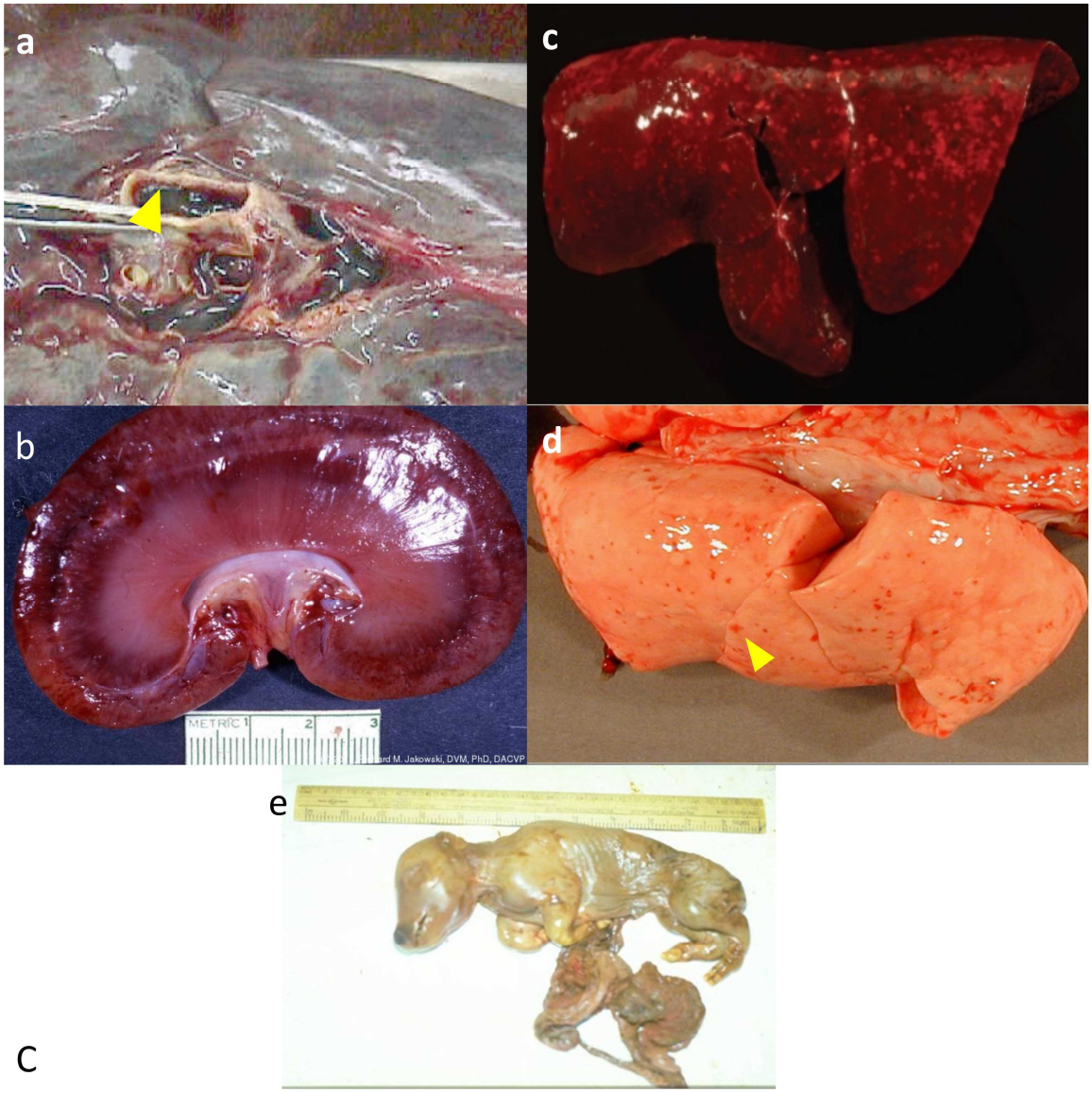
(a) Human leptospirosis with jaundice (arrowhead). [Source: modified from Michael Lowe, The Fiji School of Medicine and the Fiji School of Nursing (http://www.fsm.ac.fj/Medicine%20Website/HADIF%20website/)]

(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.

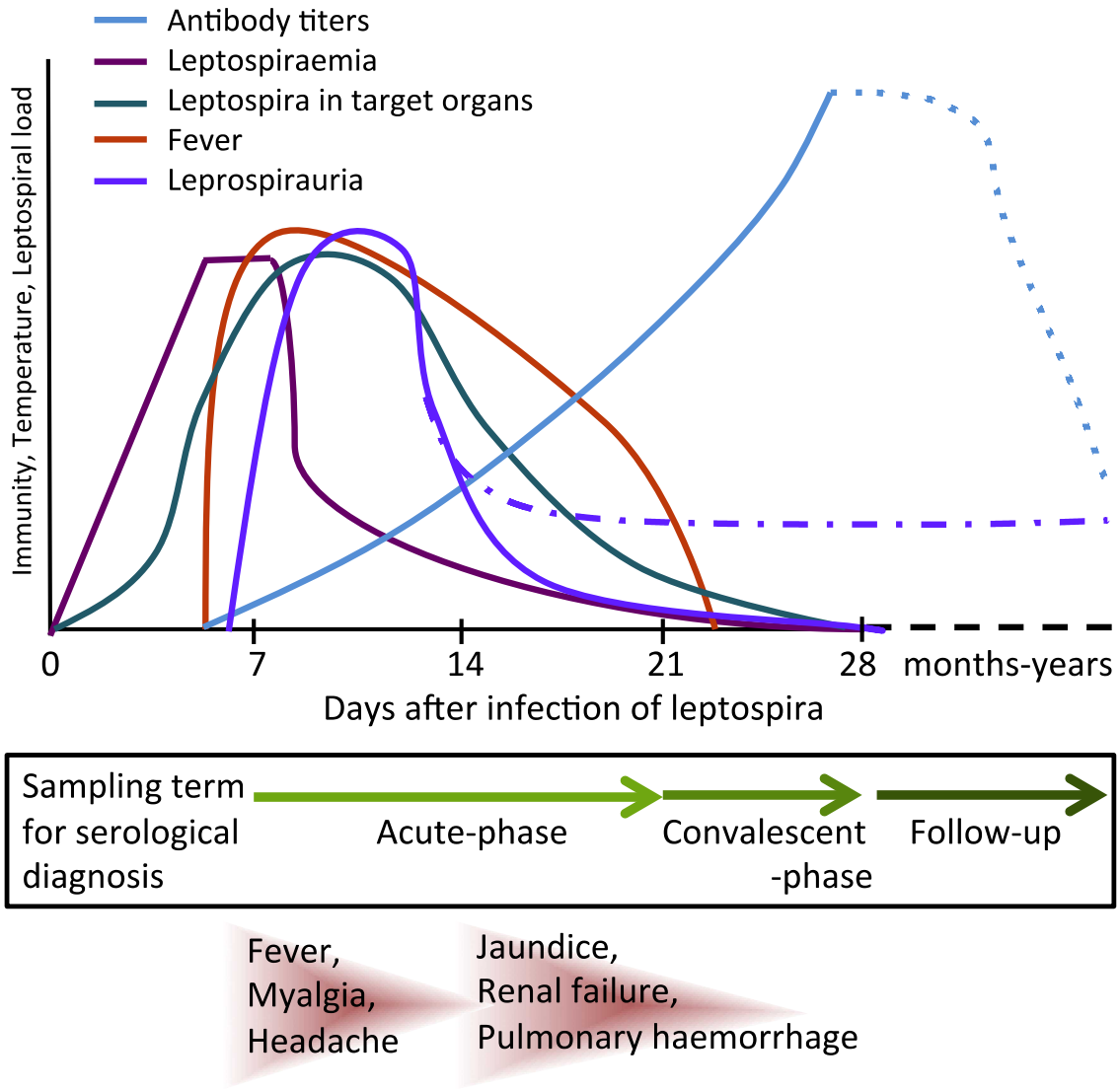


Figure 3. Leptospirosis kinetics

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).

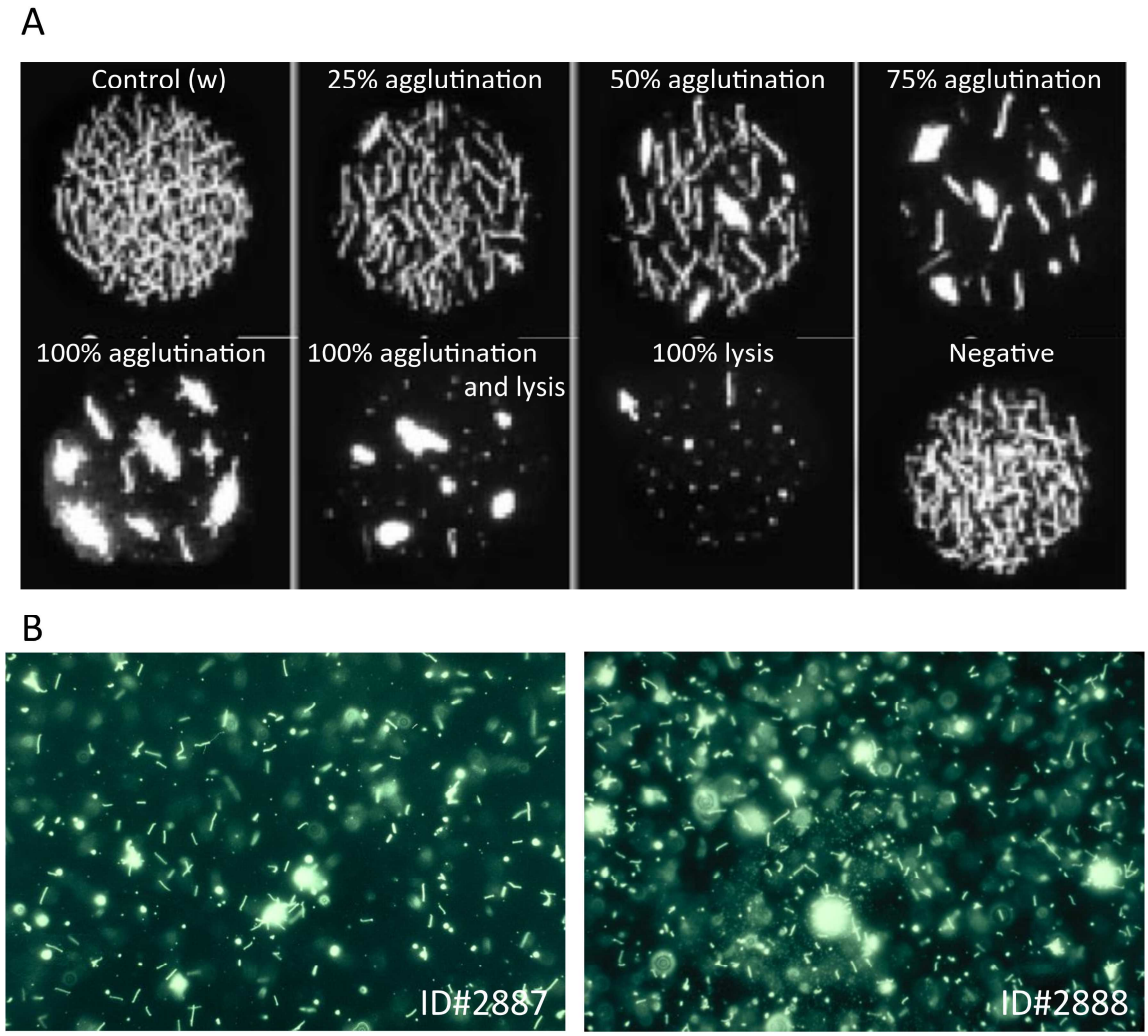


Figure 4. Microscopic agglutination test (MAT)

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.

Abbreviation

BMGY	Buffered Glycerol-complex Medium
BMMY	Buffered Methanol-Complex Medium
BSA	Bovine serum albumin
BSL	Bio Security Level
CAAT	Cross agglutinin adsorption test
DNA	Deoxyribonucleic acid
EB	ELISA Buffer
E.coli	*Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
HRP	Horseradish peroxidase
IFA	Immunofluorescence assay
IPTG	Isopropyl-ß-D-thiogalactopyranoside
kDa	Kilodalton
LB	Luria-Bertani
LFA	Lateral Flow Assay
LPHS	Leptospirosis pulmonary haemorrhage syndrome
LPS	Lipopolysaccharide
MAbs	Monoconal antibodies
MAT	Microscopic agglutination test
OD	Optical density
OPD	*o*-phenylenediamine dihydrochloride
PBS	Phosphate buffered saline
PBS-T	PBS containing 0.05% Tween 20
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
P.pastoris	*Pichia pastoris*
PVDF	Polyvinylidene difluoride
REA	Restriction endonucleases
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
SDS-PAGE	Sodium dodecyl sulfate poly-acrylamide gel electrophoresis
SIS model	Susceptibles-Infectives-Susceptibles model

tLipL32e	Truncated LipL32 expressed by *E.coli*
tLipL32p	Truncated LipL32 expressed by *P.pastoris*
WB	Western blot analysis
WHO	World Health Organization
YPD	Yeast extract peptone dextrose
YPDS	Yeast extract peptone dextrose sorbitol

Materials and Methods

Bacteria and yeast strains and culture media

Eight pathogenic *Leptospira* serovars (*L. interrogans* serovar Hebdomadis strains OP84 and Akiyami B, *L. interrogans* serovar Batavie strain Viet16, *L. interrogans* serovar Manilae strain UP-MMC-NIID, *L. interrogans* serovar Australis strains Akiyami C, *L. interrogans* serovar Autumnalis strain Akiyami A, *L. interrogans* serovar Icterohaemorrhagiae strain RGA, *L. interrogans* serovar Canicola strain Hond Utrecht IV) and a saprophytic *Leptospira* serovar (*L. biflexa* serovar Patoc strain Patoc I) were cultured at 28 ℃ in modified Korthof's medium (DENKA Seiken Co., LTD., Japan).

Escherichia coli strains BL21 (DE3) (9126, TaKaRa, Otsu, Japan) and JM109 (9052, TaKaRa) were grown at 37 ℃ in CIRCLEGROW (#3000-121, MP Biomedicals, USA) supplemented with ampicillin at 100 μg/ ml. *E.coli* strain TOP10 (C4040, Invitrogen, USA) was grown at 37 ℃ in Low Salt Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract and 0.5% NaCl, pH 7.5) supplemented with Zeocine (Invitrogen, USA) at 25 μg/ ml.

Pichia pastoris strain KM71H (K1740-01, Invitrogen) was grown at 30 ℃ in the following culture media: yeast extract peptone dextrose (YPD) medium (1% yeast extract, 2% peptone and 2% dextrose), YPDS plate (1 M sorbitol, 2% agar, and 100 μg/ml Zeocine (Invitrogen, USA) in YPD medium), buffered glycerol-complex (BMGY) medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate [pH 6.0], 1.34% yeast nitrogen base, 4 X 10^{-5}% biotin, 1% glycerol) and buffered methanol-complex (BMMY) medium (0.5% methanol in BMGY medium).

Serum and kidney samples

Laboratory rats

Twenty-four female WKAH/hkm rats (6 weeks old, SLC, Hamamatsu, Japan) were inoculated intraperitonially (i.p.) with 1 x 10^8 *L. interrogans* serovar Manilae strain UP-MMC-NIID. The number of leptospires in the culture medium was counted in a counting chamber (C-Chip, AR BROWN Co. Ltd., Tokyo, Japan) under a dark field microscope. Serum and kidney specimens were collected at days 3, 6, 8, 12, 14, 21, 30, 45, and 60 after inoculation. Eight female WKAH/hkm rats (6 weeks old, SLC, Hamamatsu, Japan) were used for controls.

Field rats

A total of 33 field rats (31 *Rattus norvegicus* and 2 *Rattus tanezumi*) were captured at Hai Phong Port, Vietnam in July 2011. A total of 107 field rats (104 *R. norvegicus* and 3 others) were captured in Hanoi City, Vietnam in November 2012 (53 *R. norvegicus* and 1 other) and in March 2013 (51 *R. norvegicus* and 2 others). Serum and kidney specimens were collected and stored at -80 ℃ until use.

Detection of the *flaB* gene by PCR

A portion of each kidney was comminuted and DNA was extracted by using DNAzol reagent according to the instructions of the manufacturer (Life Technologies Inc. USA). The extracted DNA fraction was used for nested PCR targeting the *flaB* gene as described previously[58]. The genomic DNA of a fraction from *L. interrogans* serovar Manilae strain UP-MMC-NIID was used as a positive control.

Serological analysis

Enzyme-linked immunosorbent assay (ELISA)

Ninety-six-well plates (3590, Corning, USA) were coated with recombinant LipL32 expressed by *E.coli* and *P.pastoris* (described below) or with formalin-treated leptospires at 37 ℃ for 1 h. After being washed three times with Dulbecco's phosphate buffered saline (PBS) containing 0.05% Tween 20 (PBS-T) by a microplate washer (Immuno Wash Model 1575, BioRad, USA), the plates were blocked with PBS containing 3% bovine serum albumin (BSA; Sigma-Aldrich, USA) at 4 ℃ overnight. After washing the plates three times with PBS-T, diluted rodent sera with ELISA buffer (EB; PBS containing 0.05% Tween 20 and 0.5% BSA) or culture supernatants of hybridomas were added to the plates. After incubation for 1 h at 25 ℃, the plates were washed three times with PBS-T. Bound antibodies were detected with a horseradish peroxidase (HRP)-labeled secondary antibody for 1 h at 25 ℃. After washing the plates three times with PBS-T, color reactions were performed with 100 µl of 0.17% *o*-phenylenediamine dihydrochloride (OPD) (Sigma-Aldrich, St. Louis, MO, USA) and 0.02% H_2O_2 substrate solution and were allowed to develop for 10 min at 25 ℃. The reaction was stopped by adding 30 µl of 10% sulfonic acid. Absorbance was measured at 490/650 nm by using a SpectraMax 340 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). The ELISA optical density (OD) values obtained in duplicate were averaged for the following analyses.

Following HRP-conjugated secondary antibodies, goat anti-rat IgG (Kirkegaard & Perry Laboratories, Inc. (KPL), Gaithersburg, MD, USA), goat anti-mouse IgG (KPL), and mouse anti-rat IgG (KPL) were used for the detection of antibodies from laboratory rat and field rat sera, mouse monoclonal antibodies (MAbs), and antibodies from laboratory rat sera that competed with MAbs, respectively.

Genus-specific antibody detection ELISA

The procedure used followed the guidelines published by WHO and ILS[59]. Briefly, the antigen was prepared the sterilized *L. biflexa* serovar Patoc at a density of 10^8-10^9 leptospires/ml in PBS and centrifuged at 19,800 x *g* for 30 min. Ninety-six-well plates (3590, Corning) were coated with 150 ul antigen/ well except for edge rows and columns and incubated at 37 ℃ for 1 h. The plates were washed three times with PBS-T and were blocked with PBS containing 3% BSA (Sigma-Aldrich). After washing the plates three times with PBS-T, eight control rat sera, 24 experimentally leptospires-inoculated rat sera and 140 field rat sera were duplicated and were added at a dilution of 1:200 in EB to the plates. Then the plates were incubated at 37 ℃ for 1 h and were washed three times with PBS-T. Bound antibodies were detected with HRP-conjugated goat anti-rat IgG (KPL) diluted at 1:10,000 in EB for 1 h at 37 ℃. The plates were washed three times with PBS-T and color reactions were performed with 100 μl of 0.17% OPD (Sigma-Aldrich) and 0.02% H_2O_2 substrate solution. The color reactions were allowed to develop for 10 min at 25 ℃ and were stopped by adding 30 μl of 10% sulfonic acid. Absorbance was measured at 490/650 nm by using a SpectraMax 340 microplate spectrophotometer. The results of duplicated measurements of sera were averaged for the following analyses.

Western blotting (WB)

An antigen was fractionated by 10-20% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (ePAGEL T1020, ATTO, Tokyo, Japan) and transferred to a polyvinylidene difluoride (PVDF) membrane (ATTO). The membrane was incubated overnight with 3% BSA (Sigma-Aldrich) in PBS at 4 ℃. After washing with PBS, the membrane was reacted with a solution containing the first antibody diluted in PBS or culture supernatant of hybridomas at room temperature for 1 h. Binding antibodies were detected using HRP-conjugated secondary antibodies, and 4-chloro-1-naphthol (Sigma-Aldrich) was used as a peroxidase substrate. Two kinds of HRP-conjugated secondary antibodies, goat anti-rat IgG (KPL) and Protein A (Zymed Laboratories Inc., USA), were used to detect antibodies of laboratory rats and field rats and mouse MAb,

respectively.

Preparation of monoclonal antibodies

Five-week-old female BALB/c/slc mice were injected i.p. with 100 μg of formalin-treated whole cells of *L.interrogans* serovar Australis strain Akiyami C that was mixed with an equal volume of Freund's complete adjuvant (Difco, New Jersey, USA). The mice were given booster doses using the same route. Two weeks later, the mice were injected with the same antigen mixed with Freund's incomplete adjuvant (Difco). Four weeks after the second immunization, a final injection without the adjuvant was given by the intravenous route. Three days later, spleens were removed and used for fusion to Sp2/O-Ag14 myeloma cells using polyethylene glycol 1500 (PEG 1500; Boehringer, Ingelheim, Germany) as described before[60]. First, hybridoma culture fluids were screened for the presence of leptospires-specific antibodies using the immunofluorescence assay (IFA)[61]. Next, each of the hybridomas secreting MAbs against LipL32 was examined by ELISA using recombinant LipL32 antigen as described above. Supernatants containing MAbs were recovered by centrifugation, filtered with a 0.45-μm Minisart filter (Sartorius, Germany), and stored at -80 ℃ until use.

Monoclonal antibody characterizationÉÉ

Western blotting (WB)

Reactivities of MAbs against *L. interrogans* serovar Hebdomadis strain OP84, serovar Batavie strain Viet16 and serovar Manilae strain UP-MMC-NIID, *L. biflexa* serovar Patoc strain Patoc I and recombinant LipL32 in WB were determined as described above. *Leptospira* was lysed with 1% SDS and 1% 2-mercaptoethanol. The lysate was boiled for 5 min at 98 ℃ and used as the antigen at 10^6 leptospires/lane. Culture media of hybridoma cells were used as the first antibody, and HRP-conjugated Protein A (Zymed) at 1:200 dilution was used as the secondary antibody.

Microscopic agglutination test (MAT)

The serum compliment component in examined sera was inactivated by heating at 56 ℃ for 30 min. Sera were made two-fold dilutions from one twenty-fifth in PBS. *L. interrogans* serovar Australis strain Akiyami C was used for characterization of MAbs and *L. interrogans* serovar Manilae strain MMC was used for titer measurement of

experimentally infected laboratory rat sera. The two strains were subcultured for five to seven days at 30 ℃ in Korthof medium and optimized with PBS to obtain a density of 1-2 x 10^8 leptospires/ml before being tested. Fifty ul of diluted sera and 50 ul of density-optimized leptospires were dispensed in each well of 96-well plates (3590, Corning), and the plates were covered and incubated at room temperature in the dark for 2 h. Fifty ul of PBS was used instead of a sera with density optimized leptospires for a control well and was included in each plate. After the incubation, specimens were transferred from each well to each section of a counting chamber (C-Chip). The reading of each section was determined in relation to the agglutination of the corresponding control antigen. Serum that showed an agglutination of at least 50% of the leptospires was considered positive, and the titer of serum was defined as the reciprocal of the highest dilution that was considered positive.

Enzyme-linked immunosorbent assay (ELISA)

ELISA was carried out according to the procedure described above. *L. interrogans* serovar Icterohaemorrhagiae strain RGA, *L. interrogans* serovar Canicola strain Hond Utrecht IV, *L. interrogans* serovar Autumnalis strain Akiyami A, *L. interrogans* serovar Hebdomadis strain Akiyami B, and *L. biflexa* serovar Patoc strain Patoc I were treated with formalin according to the WHO standard reference[59]. Formalin-treated leptospires at 10^8/ml and 1 μg/ml of recombinant LipL32 expressed in *E. coli* were used as antigens. Culture supernatants of hybridoma cells were used as the first antibody, and HRP-conjugated goat anti-mouse IgG (Zymed) at 1:10,000 dilution was used as the secondary antibody. The individual MAbs were isotyped in cell culture supernatants by ELISA followed by peroxidase-conjugated rabbit anti-mouse IgG1, IgG2a, IgG2b, IgG3, or IgM (Zymed), as described above.

Epitope mapping by using synthetic peptides

Peptides were synthesized and analyzed by Sigma Aldrich Japan (Custom SPOTs, 96 SPOTs). Briefly, various 10-mer peptides of 8-mer overlapping each based on the deduced amino acid sequence of LipL32 of strain Akiyami A (GenBank accession number AB094435) were spotted on a membrane. The spotted membrane was reacted with hybridoma culture supernatants and a secondary antibody against mouse IgG.

Epitope mapping by competitive binding assay

A competitive binding assay to whole LipL32 expressed by *E.coli* (wLipL32, described below) in ELISA was performed. As described above, 96-well plates were coated with wLipL32 and blocked with BSA. The plates were washed three times with PBS-T. Culture fluid of MAbs was added and incubated for 1 h at room temperature, and the plates were washed as described above. Then 1:200 dilutions of immunized rat sera prepared as described above were added and incubated for 1 h at room temperature. After washing as described above, HRP-conjugated mouse anti-rat IgG antibody (Zymed) was added to the wells and incubated for 1 h at room temperature. Color development was performed as described above by using OPD reagent.

Expression of whole and truncated LipL32 by *E. coli* and *P. pastoris*

Amplification and subcloning

The full coding region of the *lipL32* gene was amplified from genomic DNA of strain UP-MMC-NIID prepared by DNAzol reagent as described above. Primers were designed from the LipL32 sequences of serovar Manilae strain (GenBank accession number JQ013519) and serovar Lai strain (GenBank accession number AY568679). Primers were also designed with an additional sequence recognized by restriction enzymes (*Eco*RI, *Xho*I and *Not*I sites shown by underlines). To amplify the entire coding region of LipL32, forward primer lipL32_F1 (5'- CGCTCGAGATGATGAAAAAACTTCGATT (*Xho*I)) and reverse primer lipL32_R819 (5'- GGAATTCTCATTACTTAGTCGCGTGAG (*EcoR*I)) were used. After amplification, the DNA fragment was subcloned into pRSET A plasmid vector (Invitrogen) digested with *Xho*I/*Eco*RI. After cloning, the N-terminal sequence was found to be misaligned and was corrected by inverse PCR using the primer Inv_lipL32_right (5'- ATGAAAAAACTTTCGATTTTGGCTATCTCCGTTGCA) and the primer Inv_lipL32_left (5'- CTCGAGCTCGGATCCCCATCGATC (pRSET-A-wlipL32)).

To amplify truncated LipL32 of 87-188 amino acids corresponding to the nucleotide sequence from 259 to 564, forward primer tlipL32e_F259 (5'- CGCTCGAGCCTGCCGTAATCGCT (*Xho*I))) and reverse primer tlipL32e_R564 (5'- GGAATTCTCAATTAGGGATCTTGAT (*EcoR*I)) were used. After amplification, the DNA fragment was subcloned into pRSET A plasmid vector (pRSET-A-tlipL32). To amplify the same fragment for *P. pastoris* expression, forward primer tlipL32p_F259 (5'- GGAATTCCCTGCCGTAATCGCT (*EcoR*I)) and reverse primer tlipL32p_R564 (5'- ATAGTTTAGCGGCCGCATTAGGGATCTTGAT (*Not*I)) were used. After amplification,

the DNA fragment was subcloned into pPICZα A plasmid vector (PICZα-A-tlipL32) as described above. Sequences of the inserts were confirmed by DNA sequencing.

Production of recombinant antigens

The cDNAs containing the coding sequences for whole LipL32 and truncated LipL32 were subcloned into the expression plasmids pRSET A and pPICZα A and designated as pRSET-A-wlipL32, pRSET-A-tlipL32, and PICZα-A-tlipL32, respectively. Plasmid DNA constructs pRSET-A-wlipL32 and pRSET-A-tlipL32 were transformed into *E. coli* strain BL21 (DE3) (Invitrogen). A single colony was inoculated into CIRCLEGROW (#3000-121, MP Biomedicals) containing ampicillin for small-scale culture incubation at 37 ℃ overnight. The culture fluid was then centrifuged, and the collected cells were inoculated into 100 ml of fresh medium, and then isopropyl-ß-D-thiogalactopyranoside (IPTG) induction was performed according to the procedure for pET system expression (Novage, Invitrogen). The cultured cells were collected by centrifugation, resuspended in 5 ml of 0.5 M NaCl binding buffer (0.5 M NaCl, 20 mM imidazole, 20 mM potassium phosphate), and sonicated four times for 15 sec each time on ice. Thereafter, the fusion protein was purified using a His-trap column (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's instructions. The recombinant proteins expressed by pRSET-A-wlipL32 and pRSET-A-tlipL32 in *E. coli* were designated as wlipL32 and tlipL32e, respectively.

The plasmid pPICZα A-tlipL32 was propagated in *E. coli* strain TOP10 and linearized with restriction enzyme *BstX*1. Then it was transformed into *P. pastris* strain KM71H by using an EasyComp^{TM} Kit (K1740-01, Invitrogen) according to the manufacturer's instructions. Transformed KM71H was grown in 200 ml BMGY in a 1 L baffled flask by agitating at 300 rpm until OD_{600} reached at 2 to 6. The cultured *P. pastoris* was centrifuged at 140 x *g* for 5 min and the pellet was resuspended in 40 ml BMMY in a 300 ml baffled flask for further culture. The expression of truncated LipL32 was induced by addition of 100% methanol to the final concentration of 0.5% every 24 h. Recombinant protein fused with 6 x His was secreted from yeast into the culture fluid. A 0.5-ml aliquot of culture medium was collected at 0, 24, 48, 72, 96, 120 and 144 h to analyze the expression levels and the optimal time after induction of recombinant protein by SDS-PAGE and subsequent protein staining with Simply Blue Safe Stain (Invitrogen). Then large-scale culture was conducted under the optimized expression conditions, and transformed KM71H was propagated in 500 mL BMGY and resuspended in l L BMMY for 120 h under the same conditions as those for the small-scale culture. Thereafter, the

truncated LipL32 protein was purified from culture supernatant by using a His-trap column (GE Healthcare) as described above. The recombinant protein expressed by pPICZαA-tlipL32 in *P pastris* was designated as tlipL32p.

Adsorption of rodent sera with *E.coli*

E. coli strain BL21(DE3) was grown at 37 ℃ in CIRCLEGROW (#3000-121, MP Biomedicals) until the OD_{600} reached 0.8 and was harvested by centrifugation at 3,500 x *g* for 10 min. The pellet was washed with PBS 2 times and then stored at 4 ℃ until use. Sera from 24 *Leptospira*-inoculated and 8 control laboratory rats and 33 field rats from Hai Phong Port, Vietnam were used. One hundred µl of rodent serum was diluted 1:100 in EB, mixed with an equal volume of stored *E.coli* with the concentration adjusted to OD_{600} of 2.38 with EB, and incubated at room temperature for 1 h with agitation at 200 rpm. Adsorbent *E.coli* was separated by centrifugation at 3,500 x *g* for 5 min, and the supernatant was carefully transferred to a new tube. The treated and untreated sera were examined by ELISA as described above.

Statistics

ELISA data sets were analyzed by JMP software (ver. 11.0.0, Cary, North Carolina) and an online program for the Shapiro-Wilk Normality Test (available at http://sdittami.altervista.org/shapirotest/ShapiroTest.html).

Ethics statement

Animal experimentation was performed after obtaining permission from the Institutional Animal Care and Use Committee of Hokkaido University. Experiments involving infection were performed in a BSL-2 facility.

Results

Production of hybridoma clones generating MAbs against *Leptospira interrogans*

A total of 123 hybridoma clones were confirmed to produce MAbs against *L. interrogans* serovar Australis by IFA. Clones were divided into groups A to E based on cross-reactivity against 5 strains of pathogenic *Leptospira* and 1 strain of nonpathogenic *Leptospira* by ELISA, the results of the MAT against *L. interrogans* serovar Australis Akiyami C, and molecular weights of the reacted fraction from *L. interrogans* serovar Australis in WB (Table 1).

Groups of hybridoma (number of clones)	MAT	ELISA						WB (kDa)
	L. interrogans	*L. interrogans*					*L. biflexa*	*L. interrogans*
	Australis	Australis	Icterohaemorrhagiae	Canicola	Autumnalis	Hebdomadis	Patoc	Australis
A (4)	−	+	+	+	+	+	−	32
B (2)	−	+	+	+	+	+	+	40
C (115)	+	+	−	−	−	−	−	21
D (1)	−	+	−	−	−	−	−	45
E (1)	−	+	−	−	−	−	−	48

Table 1. Identification of hybridomas generating MAbs against LipL32
A total of 123 hybridoma clones were distinguished by MAT, ELISA and WB.
Only Group C maintained affinity for protease K-treated antigen.

MAbs in Group A reacted to a fraction with an estimated molecular weight of 32 kDa in WB but were negative in the MAT. They had cross-reactivity to all of the 5 strains of pathogenic *Leptospira* but not to the strain of nonpathogenic *Leptospira* in ELISA. MAbs in Group B reacted to a fraction with an estimated molecular weight of 40 kDa in WB but were negative in the MAT. They reacted with all of the pathogenic and nonpathogenic *Leptospira* strains. A total of 115 MAbs in Group C recognized a fraction with an estimated molecular weight of 21 kDa in WB and were positive in the MAT. They reacted only to homologous serovar Australis in ELISA. Group D and E contained one MAb each, which recognized fractions with estimated molecular weights of 45 and 48 kDa, respectively. They were positive only to homologous serovar Australis in ELISA.

Specificity of MAbs in Group A to LipL32 was expected, based on the cross-reactivity to pathogenic *Leptospira* but not to a nonpathogenic *Leptospira*, negative results of the

MAT, and reactivity to a protein with an estimated molecular weight of 32 kDa. Therefore, 4 MAbs designated as D14/2, D58/3, Y22/1 and D62/1were used in further characterizations.

Characterization of MAbs in Group A

Isotype of MAbs

The immunoglobulin sub-classes of MAbs D14/2, Y22/1, D58/3 and D62/1 were determined to be IgG1, IgG2a, IgG2b and IgG2a, respectively.

Specificity of MAbs in Group A

Whole LipL32 (wLipL32) was used for WB to confirm reactivity of MAbs. D58/3 reacted with wLipL32 expressed by *E. coli* (Figure 5, lane 1) and showed a single band with an estimated molecular weight of 32 kDa from *L. interrogans* serovars Manilae, Hebdomadis and Batavie (Figure 5, lanes 3, 4 and 5). No band was obtained from the lane of *L. biflexa* serovar Patoc (Figure 5, lane 2), being in accordance with the results of ELISA. The other 3 MAbs showed the same results. Thus, the specificity of MAbs (D14/2, Y22/1, D58/3 and D62/1) to LipL32 was confirmed.

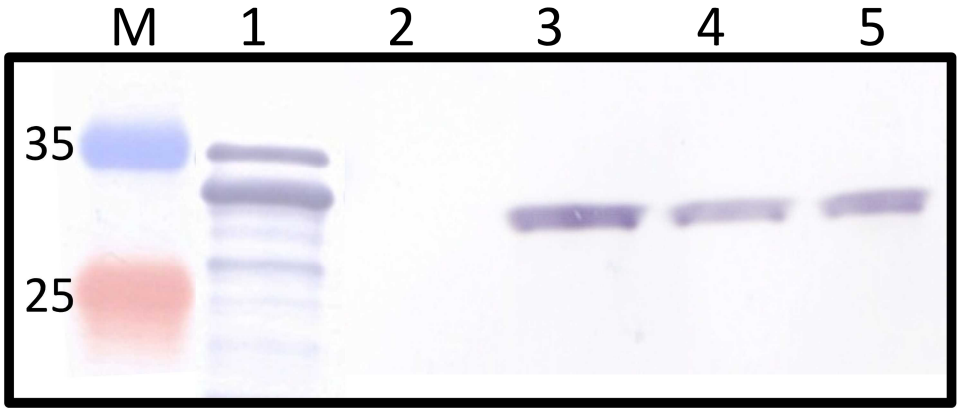


Figure 5. Antigenic specificities of the MAbs against LipL32

MAbs detected an estimated 32-kDa antigen of *L. interrogans* serovars Manilae (lane 3), Hebdomadis (lane 4) and Batavie (lane 5) but no any band in *L. biflexa* serovar Patoc strain Patoc I (lane 2). wLipL32 was detected as an antigen with an estimated molecular weight of 33~34 kDa (lane 1).

Epitope mapping

As shown in Figure 6, the amino acid sequences of epitopes for the MAbs were determined to be EIGEPGDGDL, DGDDTYKE and KIPNPPKS, corresponding to amino acid positions 105-114 (D14/2), 167-174 (D58/3 and D62/1) and 187-194 (Y22/1) of LipL32, respectively. Since D58/3 and D62/1 recognized the same amino acid sequence, only D58/3 was used in the following experiments.

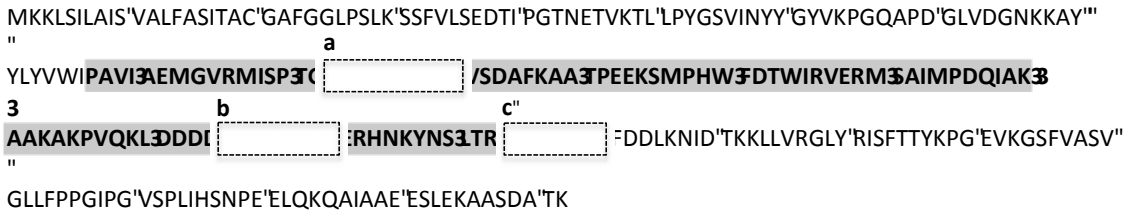


Figure 6. Epitopes of MAbs in the amino acid sequence of LipL32 in *L.* serovar Manilae

According to the results of SPOTs, epitopes of MAbs are shown in the dashed-line boxes: (a) D14/2, (b) D58/3 and D62/1, and (c) Y22/1. The gray shaded region indicates the amino acid sequence of tLipL32.

Determination of immunodominant epitopes on LipL32

A competitive assay between MAbs and polyclonal immune sera was carried out to evaluate immunodominant epitopes on LipL32. A total of 24 sera from rats experimentally inoculated with *L. interrogans* serovar Manilae were tested in ELISA. As shown in Figure 7, ELISA ODs with polyclonal immune sera were reduced by blocking epitopes with the MAb D14/2, D58/3, or Y22/1. The average reduction rate at 6 to 60 days after inoculation was highest with D58/3 (58%) and next highest with D14/2 (46%). On the other hand, Y22/1 showed an average reduction of 13%. Thus, the antigenic region covering the two epitopes of D14/2 and D58/3 is considered to be an immunodominant region.

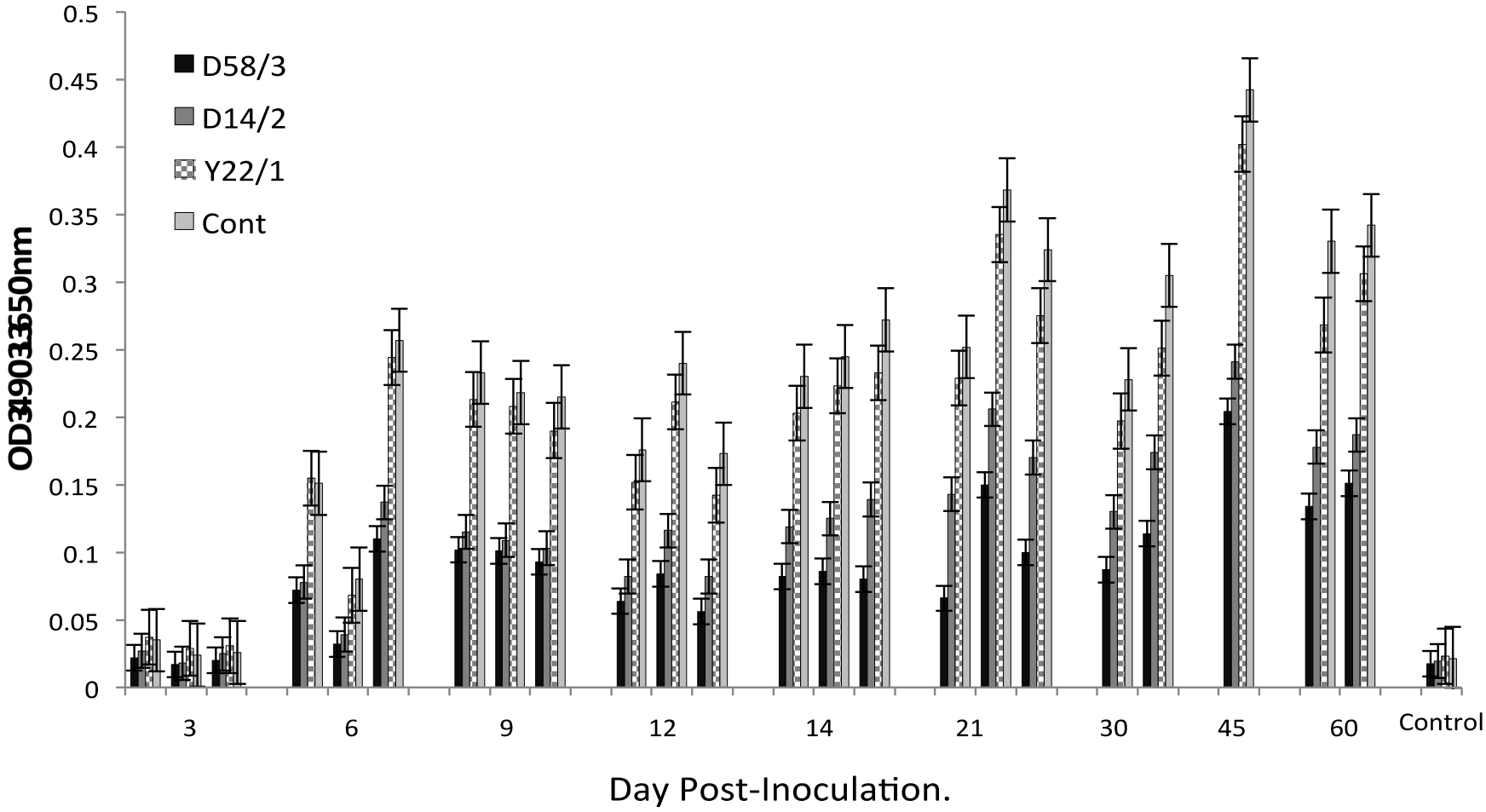


Figure 7. Competitive binding assay with MAbs and immunized rat sera

Rat sera experimentally inoculated with *L. interrogans* serovar Manilae were examined by wLipl32 ELISA. MAbs D58/3, D14/2, Y22/1 and control (invalid MAb against hantavirus) competed with polyclonal antibodies to bind to wLipL32. *Results from 8 control rat sera are shown as averaged ODs. Error bars are indicated in the figure.

Preparation of truncated recombinant LipL32

In repeated use, wLipL32 showed rapidly reduced antigenic efficacy for ELISA. This was probably due to the temperature instability of wLipL32 related to the cycles of freeze and thaw. To solve this problem, truncated LipL32 in *E.coli* (tLipL32e) and that in *P. pastoris* (tLipL32p) were developed.

wLipL32 and tLipL32e were expressed by *E.coli* BL21 (DE3) as soluble proteins, and tLipL32p was expressed by *P. pastoris* KM71H as a soluble extracellular secreted protein. The expressed proteins were purified and subjected to WB analysis using MAbs. As shown in Figure 5, wLipL32 was detected between 21~35 kDa in a ladder band. The major band was at an estimated molecular weight of 32~34 kDa (Figure 5, lane 1). tLipL32p was confirmed to be a protein with an estimated molecular weight of 12~13 kDa (Figure 8, lane 1). tLipL32e was detected with an estimated molecular weight of 15~16 kDa (Figure 8, lane 2).

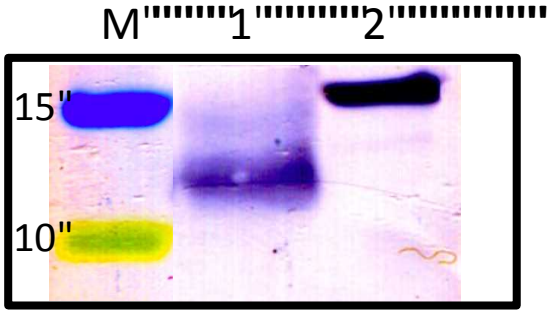


Figure 8. Antigenic specificities of the MAbs against tLipL32

D58/3 and D14/2 showed affinity to tLipL32p and tLipL32e.
D58/3 detected tLipL32p (lane 1) as an antigen with an estimated molecular weight of 12.5~13 kDa and detected tLipL32e as an antigen with an estimated molecular weight of 15~16 kDa (lane 2). Standard protein markers are shown in lane M.

Reactivity of LipL32 with rat sera

Comparison of reactivities of tLipL32 expressed by *E. coli* and *P. pastris* in ELISA with rat sera

Antigenic efficacies of tLipL32e and tLipL32p were examined by comparing reactivities with sera from field rats that were defined as following. Field rat serum that showed the highest OD value in ELISA and positive results in *flaB*-nested PCR and WB was defined as *Leptospira* infection-positive serum, and field rat serum that showed the lowest OD value in ELISA and negative results in *flaB*-nested PCR and WB was defined as

Leptospira infection-negative serum. The optimized condition of field rat sera was at a dilution of 1:200 and that of goat anti-rat IgG HRP conjugate was at a dilution of 1:10,000. As shown in Figure 8, the antigen concentrations that gave plateau OD values were 1 μg/ml for tLipL32p and 64 μg/ml for tLipL32e. tLipL32p showed high antigenic efficacy in ELISA compared to tLipL32e. Consequently, the tLipL32 ELISA condition was optimized to use 1 ug/ml tLipL32p and 8 ug/ml tLipL32e for practical antigen concentrations.

OD values with serum from an uninfected field rat indicated that tLipL32p had a very low nonspecific reaction even with higher concentrations of the antigen. tLipL32e showed high background ODs with increasing antigen concentration (Figure 9). These results also suggested that contaminated substances from the expression host *E. coli* caused a nonspecific reaction in ELISA with field rat serum.

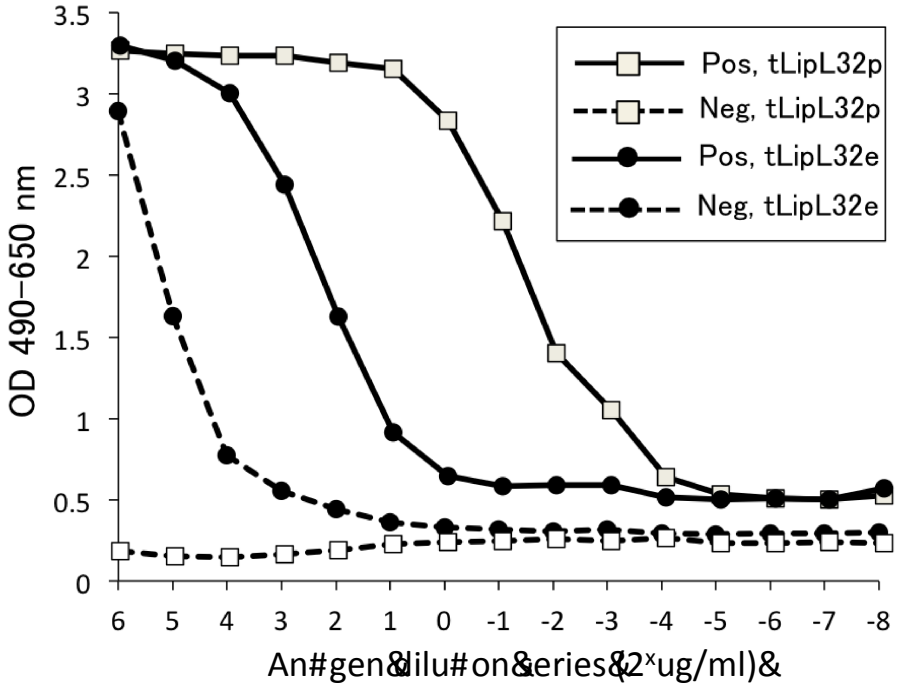


Figure 9. Optimization of antigen concentration for tLipL32 ELISA

Pos.: serum from a field rat defined as positive for leptospiral infection (solid line), Neg.: serum from a field rat defined as negative for leptospiral infection (broken line). The antigens tLipL32p (●) and tLipL32e (□) were diluted in the range of 2^{6}-2^{-8} ug/ml. A concentration of 2^{6} ug/ml of tLipL32e was required to reach an OD value saturation with the plate reader, but nonspecific reaction was observed with a concentration of more than 2^{3}-2^{4} ug/ml. A concentration of 2^{1} ug/ml of tLipL32p to reach an OD value saturation with the plate reader without showing any nonspecific reactions.

Evaluation of the effects of *E.coli*-adsorbed sera on ELISA

To reduce non-specific reaction in ELISA, rat sera were pretreated with *E.coli* to adsorb substances in sera that reacted against components of *E.coli*. A total of 33 field rat sera were adsorbed with *E. coli* as described in Materials and Methods. At first, tLipL32p was used for testing pretreated and untreated sera. As shown in Figure 10A, the correlation function between pretreated and untreated sera was R^2=0.95 (P<0.001). Next, tLipL32e was used for testing pretreated and untreated sera. As shown in Figure 10B, the distribution of ELISA OD values showed a decrease of ODs with pretreated sera compared to those with untreated sera. These results indicated that pretreatment of serum with *E. coli* was effective to reduce background reaction only for an *E. coli*-expressed antigen such as tLipL32e.

Similar experiments were performed in laboratory rats (Figure 11A and B). A total of 24 sera from laboratory rats experimentally inoculated with *L.interrogans* serovar Manilae and 8 control sera from uninfected laboratory rats were examined by ELISA. In Fig. 6A, OD values with the two antigens tLipL32p and tLipL32e were strongly correlated with two exceptions of high ODs to tLipL32e but lower to tLipL32p. As shown in Fig. 6B, no significant reduction of non-specific reaction was observed with *E.coli*-adsorbed sera. Both in Fig. 11A and 11B, the distribution of OD values for 8 control rats was found lower and was separately from rats inoculated with leptospires. These results suggested that both tLipL32p and tLipL32e are applicable as ELISA antigens for laboratory rat sera.

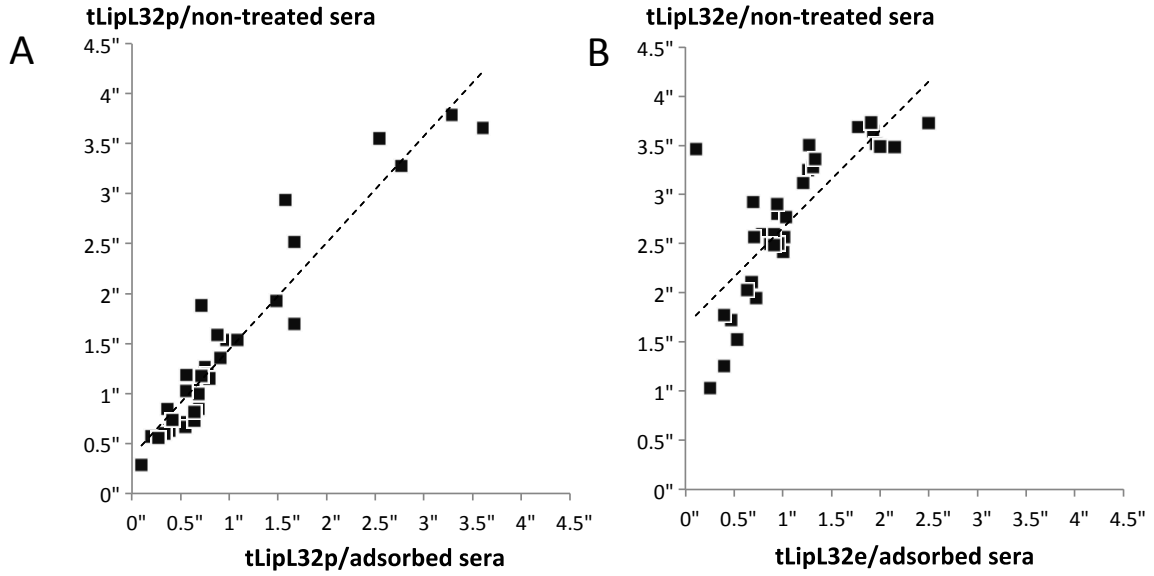


Figure 10. Comparison of tLipL32p and tLipL32e antigens and effects of serum pretreatment by *E. coli* adsorption with field rat sera

E.coli adsorption pretreatment of sera did not have an effect on tLipL32p-ELISA, but ODs were reduced by *E.coli* adsorption pretreatment of sera for tLipL32e-ELISA.

A: tLipL32p-ELISA with field rat sera and tLipL32p-ELISA with field rat sera pretreated by *E.coli* adsorption

B: tLipL32e-ELISA with field rat sera and tLipL32e-ELISA with field rat sera pretreated by *E.coli* adsorption

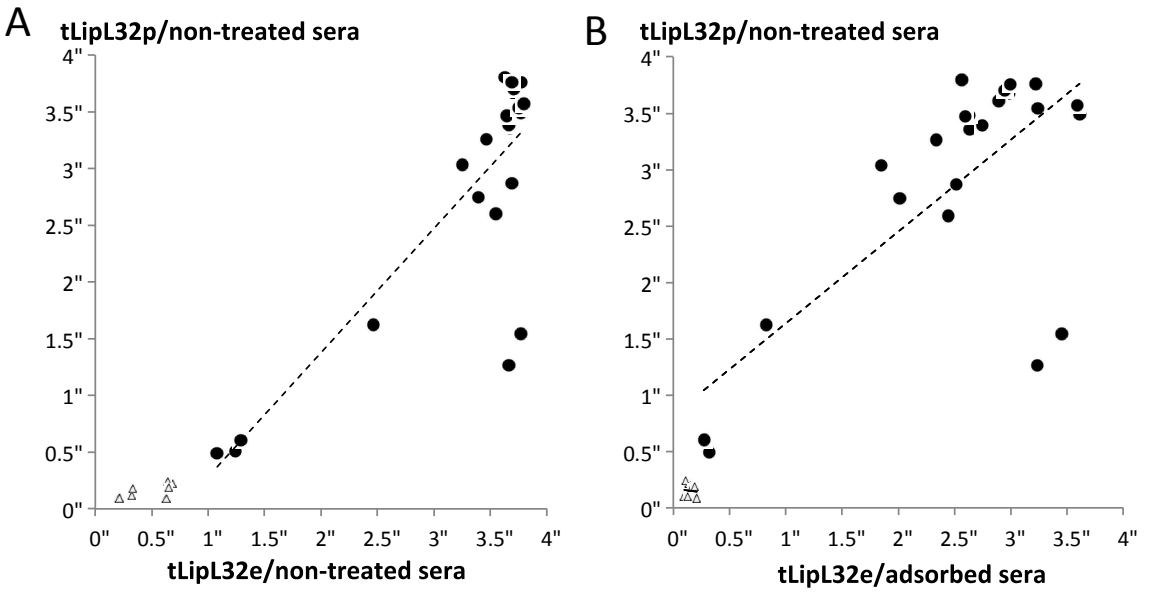


Figure 11. Comparison of tLipL32p and tLipL32e antigens and effects of serum pretreatment by *E. coli* adsorption with experimentally inoculated rat sera

Open triangles show laboratory rats without inoculation (control rat sera).

A: tLipL32p-ELISA and tLipL32e-ELISA with experimentally inoculated and control rat sera.

B: tLipL32p-ELISA with experimentally inoculated and control rat sera, and tLipL32e-ELISA with experimentally inoculated and control rat sera pretreated by *E.coli* adsorption.

Evaluation of tLipL32 ELISA for field rat sera

Generally, to evaluate a novel diagnosis system, true positive and true negative groups of sera are required. However, it was difficult to identify true negative rats among field rats. Therefore, true positive sera and true negative sera groups were tentatively defined on the basis of results from *flaB* gene PCR and WB with whole *Leptospira* cells. A total of 33 field rat sera were divided into four groups: PCR alone positive, WB alone positive, PCR and WB positive, and PCR and WB negative (Figure 12). Among them, sera in the PCR and WB positive group were tentatively regarded as true positive, and sera in the PCR and WB negative group were tentatively regarded as true negative.

The distribution ranges of OD values in ELISA using tLipL32 were compared in the true positive and true negative groups were compared. A tentative cutoff point for tLipL32 ELISA was defined as 95% of the OD values among sera in the true negative group, so as to have 95% specificity[51]. Sensitivity was defined as the percentage of sera in the true positive group for which OD values were greater than the cutoff value. As shown in Figure 12A, tLipL32p could differentiate true positive and true negative groups. With the tentative cutoff point, 83% sensitivity was obtained by tLipL32p ELISA. A similar distribution range of OD values was obtained from ELISA using tLipL32e with adsorbed sera (Figure 12B), and the sensitivity was 92%. However, as shown in Figure 12C, tLipL32e with untreated sera resulted in OD values of sera in the true positive group being overlapped with ODs of sera in the true negative group and the sensitivity was 50%.

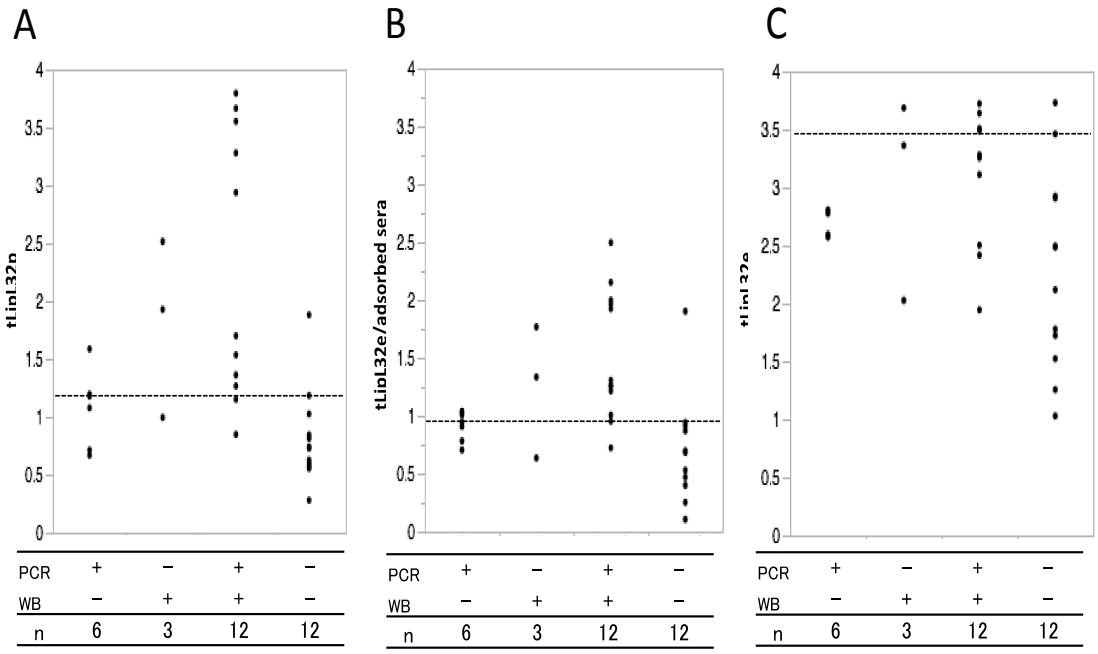


Figure 12. Distribution of ELISA ODs obtained from tLipL32 ELISA in relevance to *flaB*-nested PCR and WB

A total of 33 field rat sera were divided into four groups according to the results of PCR and WB using *L. interrogans* serovar Manilae as an antigen. Tentatively, rat sera positive by both PCR and WB were regarded as true positive and rat sera negative by both PCR and WB were regarded as true negative. A tentative cutoff points for antigens which was defined as OD values for 95% of the true negative group, is shown by a broken line.

A: Distribution of ODs from tLipL32p-ELISA with non-adsorbed field rat sera

B: Distribution of ODs from tLipL32e-ELISA with adsorbed field rat sera

C: Distribution of ODs from tLipL32e-ELISA with non-adsorbed field rat sera

Genus-specific antibody detection ELISA

Genus-specific antibody detection ELISA is an alternative method to the MAT for serological diagnosis in the early stage of human leptospirosis. The cut off point was OD 0.3, which was derived from ELISA ODs of control rat sera (average + 2 standard deviations).

The heat-treated *L. biflexa* serovar Patoc cell antigen for ELISA in this study showed reliable results among rat sera at 6 to 9 days after inoculation with *L. interrogans* serovar Manilae, with sensitivity of 92%. On the other hand, 95% of the field rat sera were positive for *Leptospira* infection (Figure 13).

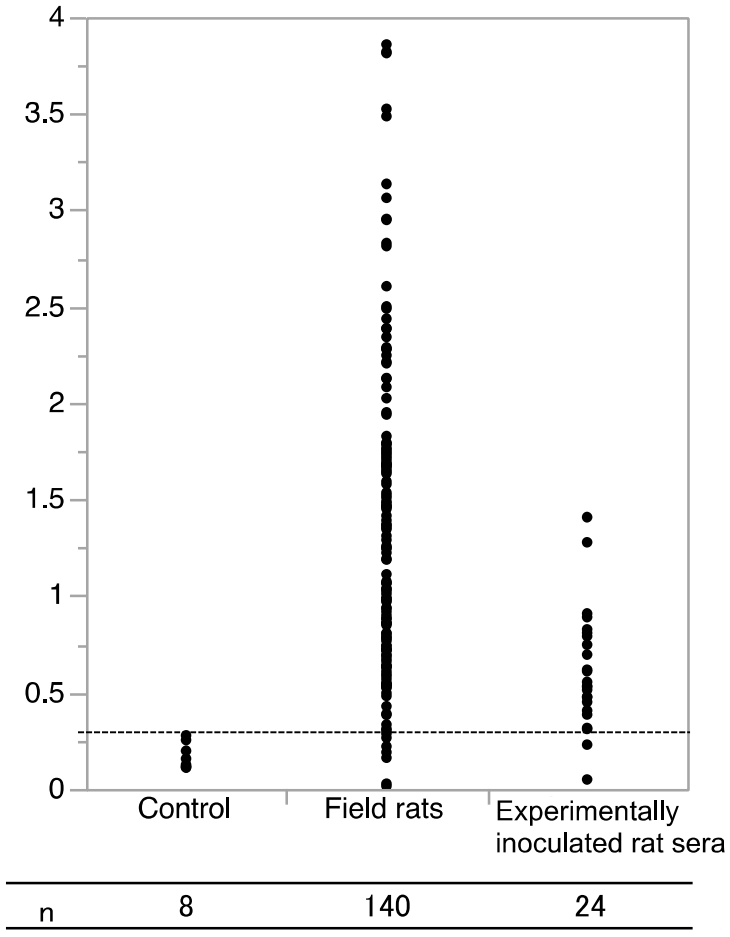


Figure 13. Genus-specific antibody detection ELISA

Eight control rat sera, 24 experimentally *leptospira* inoculated rat sera and 140 field rat sera were examined against *L. biflexa* serovar Patoc by ELISA. The sensitivity was 92% among experimentally *Leptospira* inoculated rat sera. Ninety-five percent of field rat sera were positive with the cutoff point (0.3) derived from ELISA ODs of control rat sera. The cutoff point is indicated as a broken line.

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[62-64]. 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[65-68]. 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*[65]. 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[69]. 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[70]. 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 [57,69]. *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 [71]. 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[69]. *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[59]. 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[70]. 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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