การพัฒนาวิธีการตรวจวินิจฉัยแอนติเจนของเชื้อเลปโตสไปรา ในปัสสาวะด้วยวิธีอิมมูโนฟลูออเรสเซนต์โดยใช้ โมโนโคลนอลแอนติบอดีจำเพาะต่อตัวเชื้อ

จินตภา เสือสวย[1*] ชวิกานต์ บุญวงศ์[2*] ณหฤทัย อินต๊ะสิน[1]
สุทธิชา มาตำนาน[2] ปฏิมาพร วงษ์พรหมพิทักษ์[1] และ ปัทมา เอกโพธิ์[2]

[1]*ภาควิชาวิทยาภูมิคุ้มกัน คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล*
[2]*ฝ่ายวิจัย คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล*

บทคัดย่อ

เลปโตสไปโรซิส เป็นโรคติดเชื้อจากสัตว์สู่คนที่มีอันตรายถึงชีวิต การวินิจฉัยโรคในระยะเฉียบพลันยังไม่ดีพอ เนื่องจากมีอาการป่วยที่คล้ายกับโรคติดเชื้ออื่น อีกทั้งวิธีการตรวจทางห้องปฏิบัติการที่เป็นมาตรฐาน ได้แก่ การเพาะเลี้ยงเชื้อ หรือ วิธี microscopic agglutination test (MAT) มีความไวต่ำ ในการศึกษานี้คณะผู้วิจัยได้ผลิตโมโนโคลนอลแอนติบอดี (monoclonal antibody; MAb) ที่จำเพาะกับเชื้อเลปโตสไปรา ถึง 24 serovars ประกอบด้วย serovars Australis, Autumnalis, Ballum, Bangkok, Bataviae, Bratislava, Canicola, Cynopteri, Djasiman, Grippotyphosa, Hardjo, Hebdomadis, Icterohaemorrhaegiae, Louisiana, Pomona, Pyrogenes, Ranarum, Saigon, Sarmin, Sejroe, Shermani, Tarassovi, Wolffi, และ Patoc โดย MAb ที่ผลิตได้เป็นชนิด immunoglobulin M Kappa (IgMK) ที่จำเพาะกับแอนติเจนที่ตัวเชื้อ และแอนติเจนที่เชื้อปล่อยออกมา โดยไม่ทำปฏิกิริยากับเชื้อแบคทีเรียชนิดอื่น คือ *Aeromonas hydrophila, Escherichia coli, Klebsiella pneumoniae, Proteus* spp., *Pseudomonas aeruginosa, Salmonella* spp., *Shigella* spp. และ *Streptococcus* spp. เมื่อนำ MAb นี้มาพัฒนาเป็นชุดทดสอบ MAb-based immunofluorescence assay (MAb-IFA) พบว่าสามารถตรวจหาเชื้อเลปโตสไปราได้ 24 serovars และไม่ทำปฏิกิริยากับเชื้อแบคทีเรียอื่นทั้ง 8 ชนิดข้างต้น โดยชุดทดสอบนี้สามารถตรวจหาเชื้อปริมาณน้อยที่สุดที่ 1.5 ตัวต่อไมโครลิตร ดังนั้นในการตรวจใช้สิ่งส่งตรวจ 20 ไมโครลิตร จะต้องมีเชื้อเลปโตสไปราอย่างน้อย 30 ตัว ข้อดีของวิธี MAb-IFA คือ ราคาถูก มีความไว สามารถนำไปประยุกต์ใช้เป็นวิธีทดสอบในห้องปฏิบัติการทั่วไป เพื่อช่วยวินิจฉัยโรคเลปโตสไปโรซิส ซึ่งการวินิจฉัยได้อย่างรวดเร็ว และแม่นยำ จะทำให้เกิดผลดีต่อการรักษา

คำสำคัญ: โรคเลปโตสไปโรซิส การตรวจวินิจฉัยด้วยวิธีอิมมูโนฟลูออเรสเซนต์โดยใช้โมโนโคลนอลแอนติบอดี การตรวจหาแอนติเจน การวินิจฉัย

***ผู้รับผิดชอบบทความ E-mail address:** Jintapa.sus@mahidol.ac.th, Chawikan.boo@mahidol.ed
รับบทความ: 1 มีนาคม 2562 แก้ไขบทความ: 8 มิถุนายน 2562 รับตีพิมพ์บทความ: 30 ตุลาคม 2562

Development of Monoclonal Antibody-Based Immunofluorescence Assay for Detection of Leptospires in Urine

Jintapa Sueasuay[1*], Chawikan Boonwong[2*], Naharuthai Inthasin[1], Sutthicha Matamnan[2] Patimaporn Wongprompitak[1] and Pattama Ekpo[2]

[1]*Department of Immunology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand*
[2]*Research Division, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand*

Abstract

Leptospirosis is a common life-threatening zoonotic disease. The disease is often underdiagnosed, especially in the acute phase, due to variations in presenting symptoms, some of which may be mistaken for other diseases. The low sensitivity gold standard culture isolation is a microscopic agglutination test (MAT). In this study, a hybridoma secreting monoclonal antibody (MAb) broadly specific to 24 *Leptospira* serovars including serovars Australis, Autumnalis, Ballum, Bangkok, Bataviae, Bratislava, Canicola, Cynopteri, Djasiman, Grippotyphosa, Hardjo, Hebdomadis, Icterohaemorrhaegiae, Louisiana, Pomona, Pyrogenes, Ranarum, Saigon, Sarmin, Sejroe, Shermani, Tarassovi, Wolffi, and Patoc of the *Leptospira* genus was produced. The MAb, named immunoglobulin M kappa (IgMK), reacted to secretory and whole cell antigens of *Leptospira* spp., but not with the following 8 bacterial genera: *Aeromonas hydrophila, Escherichia coli, Klebsiella pneumoniae, Proteus* spp., *Pseudomonas aeruginosa, Salmonella* spp., *Shigella* spp., and *Streptococcus* spp. A MAb-based immunofluorescence assay (MAb-IFA) subsequently developed reacted to all of the 24 known serovars of *Leptospira,* but not with the 8 aforementioned bacterial genera. A minimum of 1.5 leptospires/uL or at least 30 leptospires in 20 uL of sample volume is required for *Leptospira* identification by Mab-IFA. The MAb-IFA is a sensitive and low-cost antigen detection assay that can be performed in a routine clinical laboratory. Early and accurate diagnosis of leptospirosis will facilitate improved care and better patient outcomes.

Keywords: Leptospirosis, Monoclonal antibody-based immunofluorescence assay, Antigen detection, Diagnosis

***Corresponding author E-mail address:** Jintapa.sus@mahidol.ac.th, Chawikan.boo@mahidol.edu

Received: 1 March 2019 Revised: 8 June 2019 Accepted: 30 October 2019

Introduction

Leptospirosis is an emerging infectious disease of worldwide distribution that is caused by pathogenic spirochetes of genus *Leptospira*[1, 2]. Infection occurs either from direct exposure to infected animals or their products, or indirectly via contact with water or soil contaminated with urine of a *Leptospira* shedder[1, 3]. The symptoms of leptospirosis are generalized and they vary from flu-like illness to fatal hemorrhagic forms with severe involvement of vital organs, including the liver, lung and kidney[4-9]. The non-specific nature of the clinical symptoms associated with leptospirosis infection makes it difficult to differentiate from other prevalent and endemic infectious diseases, such as dengue virus infection, malaria, melioidosis and scrub typhus[1]. Early and accurate diagnosis of leptospirosis is important for prompt and appropriate treatment[10]. The gold standard methods for detecting pathogenic *Leptospira* spp. are culture isolation (CI) and microscopic agglutination test (MAT). However, these methods have low sensitivity, poor reproducibility, and they are time consuming[10, 11]. There have been several attempts to employ antibody detection methods for the diagnosis of acute leptospirosis[11]. However, the detection sensitivity in acute-phase samples was found to be unsatisfactory. Molecular methods, including DNA-DNA hybridization, polymerase chain reaction (PCR), and real-time PCR, have also been used for *Leptospira* detection[11]. These molecular techniques are both sensitive and specific, and they have high potential for early diagnosis of leptospirosis. However, these methods may be unaffordable in many care settings due to the need for specialized equipment and expensive reagents. Specific antigen detection, which indicates a definite diagnosis, may be an effective diagnostic method in acute leptospirosis since the antigen can be detected early after infection[12]. The MAb-based immunofluorescence assay (MAb-IFA) is a test of choice because it is a fast, sensitive, and low-cost antigen detection assay that can be performed in a routine clinical laboratory. In this study, a hybridoma secreting monoclonal antibody (MAb) broadly specific to *Leptospira* spp. was produced. A MAb-IFA was then developed to facilitate antigen detection, with subsequent testing and analysis to evaluate MAb-IFA sensitivity and specificity.

Materials and methods

The study was conducted at the Department of Immunology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand. The protocol of *Leptospira* culture for this study was approved by Siriraj Biosafety Risk Management Taskforce, Mahidol University (SI 2015-007)

Bacteria

The 24 serovars in 19 serogroups (serogroup Australis; Australis, Bangkok,

Bratislava, serogroup Autumnalis; Autumnalis, serogroup Ballum; Ballum, serogroup Bataviae; Bataviae, serogroup Canicola; Canicola, serogroup Cynopteri; Cynopteri, serogroup Djasiman; Djasiman, serogroup Grippotyphosa; Grippotyphosa, serogroup Sejroe; Hardjo, Sejroe, Wolffi, serogroup Hebdomadis; Hebdomadis, serogroup Icterohaemorrhagiae; Icterohaemorrhaegias, serogroup Louisiana; Louisiana, Saigon, serogroup Pomona; Pomona, serogroup Pyrogenes; Pyrogenes, serogroup Ranarum; Ranarum, serogroup Sarmin; Sarmin, serogroup Shermani; Shermani, serogroup Tarassovi; Tarassovi, serogroup Semaranga; Patoc) used in this study were generously provided by the National Institute of Animal Health (NIAH), Bangkok, Thailand. The other 8 bacterial genera (*Aeromonas hydrophila, Escherichia coli, Klebsiella pneumoniae, Proteus* spp., *Pseudomonas aeruginosa, Salmonella* spp., *Shigella* spp., and, *Streptococcus* spp.) used in this study were kindly provided by Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand. Each genus was obtained from 4 clinical specimens from patients hospitalized in Siriraj Hospital, Bangkok, Thailand.

Preparation of antigens

Secretory (SC) and whole cell (WC) antigens of leptospires and other bacteria were prepared according to a previously described protocol[13].

Ethics statement

All procedures and experiments involving animals were performed in accordance with the Ethical Principles for the Use of Animals for Scientific Purposes of the National Research Council of Thailand (NRCT).

MAb production and characterization

MAb was produced as previously described[13]. The specificities of MAbs were monitored by indirect enzyme-linked immunosorbent assay (ELISA) using a panel of SC and WC antigens prepared from the 24 leptospires, and WC antigens prepared from the 8 other bacterial genera. The hybrids that produced antibody broadly specific only to leptospires were identified and cloned three times by limiting dilution. MAb isotypes were determined by indirect ELISA[14]. The MAb was further tested by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and an immunoblotting method with WC and SC antigens. Partial purification of the MAb was performed by 50% ammonium sulphate precipitation method[13]. The establishment of the hybridoma was in 2004.

SDS-PAGE and immunoblotting

SDS-PAGE of the WC and SC antigens, the immunoblot reaction with the MAb, and the biochemical nature of the specific epitope were all performed as previously described[13].

Sensitivity and specificity of the MAb-based immunofluorescence assay (MAb-IFA)

A slide was coated twice with 20 µL of urine sample, with drying between coats. After the slide was fixed with cold absolute acetone for 15 min., 20 µL of partially purified MAb at a concentration of 2.0 mg/mL in 1% bovine serum albumin-phosphate buffered saline (BSA-PBS, pH 7.4; Sigma, St. Louis, MO) was added and incubated in a humidified chamber at 37°C for 20 min. The slide was washed by stirring in PBS three times (5 min for each wash), and then covered with 20 µL of an optimized dilution (1/100) of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgM antibody in BSA-PBS. The slide was incubated in a humidified chamber at 37°C for 30 min, and then washed again 3 times in PBS for 5 min for each wash. A coverslip was mounted with a mounting medium, after which the slide was examined under a fluorescence microscope (Nikon Instruments Inc., Melville, NY).

To determine the sensitivity of the test, 20 µL of *Leptospira* spiked urine at concentrations that varied from 500 to 4,000 cells/mL was coated twice onto a glass slide, with drying between coats. Non-spiked urine was used as a negative control. Aliquots of partially purified MAb that were maintained at 4°C, -20°C, and -80°C were used at different times to evaluate the sensitivity and specificity of the MAb-IFA. Specificity testing with the 24 leptospires and other 8 bacterial antigens was used for characterization of the MAb.

Results

Characterization of MAb

SC antigen prepared from *L. interrogans* serovar Autumnalis was immunized into BALB/cJ mice. A number of hybridomas that were secreting antibodies reactive with SC and WC antigens of leptospires were obtained. These MAbs were analyzed for their specificity by indirect ELISA with bacterial WC and SC antigens prepared from 24 serovars (Australis, Autumnalis, Ballum, Bangkok, Bataviae, Bratislava, Canicola, Cynopteri, Djasiman, Grippotyphosa, Hardjo, Hebdomadis, Icterohaemorrhaegiae, Louisiana, Pomona, Pyrogenes, Ranarum, Saigon, Sarmin, Sejroe, Shermani, Tarassovi, Wolffi and Patoc), and WC antigen prepared from *Aeromonas hydrophila, Escherichia coli, Klebsiella pneumoniae, Proteus* spp., *Pseudomonas aeruginosa, Salmonella* spp., *Shigella* spp., and *Streptococcus* spp. (Table 1). The isotype of this MAb is IgMK. The specific reaction of MAb to a representative *L. interrogans* serovar Bratislava, serogroup Australis WC and SC antigens was shown by immunoblotting (Fig. 1). The MAb specific reactive component in both WC and SC antigens which correspond to the smearing bands of lipopolysaccharide on SDS-PAGE silver staining was observed. The immunoreactive component specific to the MAb is carbohydrate in nature since the specific components disappeared after being treated with sodium periodate (Fig. 2). SDS-PAGE determined the decision to use WC antigen that was treated or untreated with proteinase K and

Table 1 Enzyme-linked immunosorbent assays (ELISA) of the panel antigens reacted with the MAb.

Bacterial Ag	AUS		AUT		BAL		BKK		BAT		BRA		CAN		CYN		DJA		GRI	
Type of Ag	WC	SC	WC	SC	WC	SC	WC	SC	WC	SC	WC	SC	WC	SC	WC	SC	WC	SC	WC	SC
OD.	1.2	0.5	0.7	0.3	>3.0	1.3	1.2	0.5	>3.0	1.3	0.7	0.3	1.3	0.4	>3.0	1.4	2.5	0.3	>3.0	1.2
Bacterial Ag	HAR		HEB		ICT		LOU		POM		PYR		RAN		SAI		SAR		SEJ	
Type of Ag	WC	SC	WC	SC	WC	SC	WC	SC	WC	SC	WC	SC	WC	SC	WC	SC	WC	SC	WC	SC
OD.	0.3	0.5	>3.0	1.7	2.9	2.1	2.9	1.5	0.9	0.7	0.4	0.3	0.3	0.3	>3.0	1.4	>3.0	>3.0	1.6	1.6
Bacterial Ag	SHE		TAR		WOL		PAT		*A. hydrophila*		*E. coli*		*K. pneumoniae*		*Proteus* spp.		*P. aeruginosa*		*Salmonella* spp.	
Type of Ag	WC	SC	WC	SC	WC	SC	WC	SC	WC	SC	WC	SC	WC	SC	WC	SC	WC	SC	WC	SC
OD.	0.7	0.4	2.0	2.0	0.4	0.3	0.2	0.2	0.08	-	0.07	-	0.07	-	0.06	-	0.03	-	0.05	-
Bacterial Ag	*Shigella* spp.		Streptococcus spp.		Mean Without MAb		Mean Without conjugate		Mean Without substrate		Without Ag									
Type of Ag	WC	SC	WC	SC	WC	SC	WC	SC	WC	SC	WC	SC								
OD.	0.06	-	0.07	-	0.03	0.02	0.03	0.02	0.01	0.01	0.07	0.05								

Specificity of the MAb to leptospires by Enzyme-linked immunosorbent assays (ELISA). The panel antigen prepared from 24 leptospires and 8 other bacterial genera were used in the ELISA. WC (whole cell), SC (secretory cell), OD (optical density), AUS (Australis), AUT (Autumnalis), BAL (Ballum), BKK (Bangkok), BAT (Bataviae), BRA (Bratislava), CAN (Canicola), CYN (Cynopteri), DJA (Djasiman), GRI (Grippotyphosa), HAR (Hardjo), HEB (Hebdomadis), ICT (Icterohaemorrhaegiae), LOU (Louisiana), POM (Pomona), PYR (Pyrogenes), RAN (Ranarum), SAI (Saigon), SAR (Sarmin), SEJ (Sejroe), SHE (Shermani), TAR (Tarassovi), WOL (Wolffi), PAT (Patoc). The ELISA reactions that do not add MAb, conjugate, substrate, or antigen are named without-MAb, -conjugate, -substrate and –antigen, respectively. These reactions are the negative control. The positive control was the *Leptospira* antigen reacted with its corresponding mouse polyclonal antibody. The OD of positive control used antibody at dilution 1: 1000 is > 1.0.

sodium periodate, blotted onto nitrocellulose membrane, and then probed with the MAb. The results showed that the MAb reacted with both proteinase K treated and untreated antigens; however, it did not react with the sodium periodate treated antigen. This MAb was then used in IFA for leptospiral antigen detection. The sensitivity and specificity of the MAb-IFA were determined.

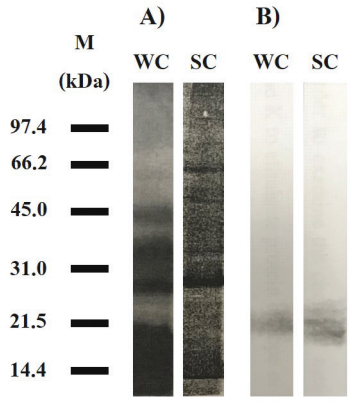

Fig. 1 Characterization of IgM MAb by immunoblotting. Whole cell (WC) and secretory (SC) antigens prepared from *L. interrogans* serovar Bratislava (serogroup Australis) were used in SDS-PAGE silver staining (A) and immunoblotting of the reaction with MAb (B).

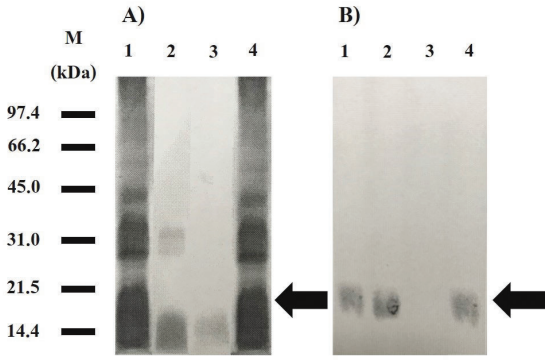

Fig. 2 Characterization of *Leptospira* biomolecule specific IgM MAb by immunoblotting. Whole cell (WC) antigen prepared from *L. interrogans* serovar Bratislava (serogroup Australis) was used in SDS-PAGE silver staining (A), and immunoblotting of the reaction with the MAb (B). The antigen was treated with proteinase K at 60°C for 1 hr, after which the enzymatic activity was stopped by incubating in boiling water for 5 min (lane 2), with its untreated control in lane 1. The antigen was also treated with sodium periodate at 4°C for 18-24 hr (lane 3), with its untreated control in lane 4. MW markers are indicated. The smearing bands of lipopolysaccharide on SDS-PAGE which correspond to the specific reactive component of the MAb are indicated as arrow.

Sensitivity and specificity of MAb-IFA, and stability of the MAb

The sensitivity of the MAb-IFA reaction with *Leptospira* spiked urine samples was determined (Fig. 3). The MAb-IFA could react with leptospires at a minimum concentration of 1.5 leptospires/ μL or 1,500 leptospires/mL. The specificity of the MAb-IFA was tested using the same antigen panel used for MAb characterization. The results showed the specificity of the MAb-IFA and the MAb to be the same. The observed reactions occurred only with leptospiral serovars. Regarding the stability of the MAb, we found that it could be maintained at 4°C, -20°C, and -80°C for at least 12 months with no observed decrease in specificity or sensitivity.

Discussion

Leptospirosis is a reemerging infectious disease, and the annual incidence and mortality rates associated with this disease are high[2, 4]. Patients with delayed leptospirosis diagnosis often receive inappropriate treatment, and this can adversely affect patient outcomes. It is difficult to distinguish leptospirosis from other diseases based on clinical symptoms alone. Therefore, laboratory confirmation plays an important role in the diagnosis of this condition. The current gold standard methods for diagnosing leptospirosis are culture isolation (CI) and microscopic agglutination test (MAT). However, these diagnostic methods are time and labor intensive[10, 11]. Moreover, the sensitivity of these tests is too low to be useful for diagnosis of this disease. Alternative diagnostic methods were developed, and most of them were antibody detection methods[11, 15, 20]; however, the sensitivity of these tests at the early phase of infection was found to be unacceptably low[11, 15]. DNA detection, such as DNA hybridization, PCR, and real-time PCR,

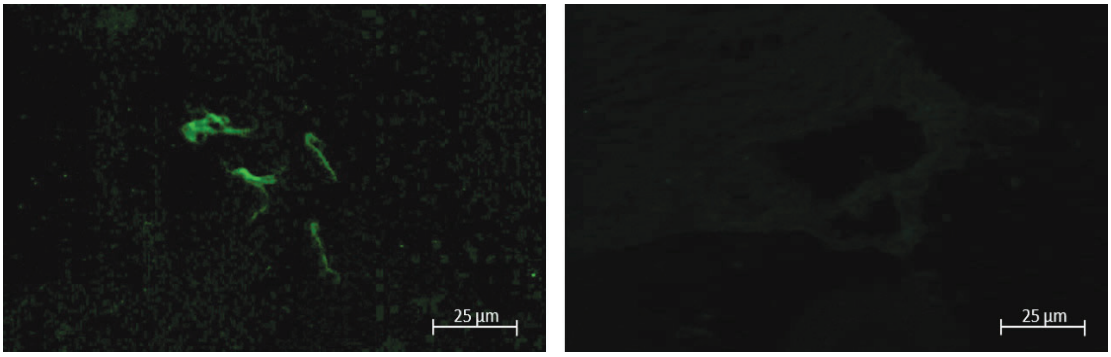

Fig. 3 Immunofluorescence assay (IFA) of fixed leptospires by using *Leptospira* specific IgM MAb. MAb-IFA of urine spiked L. interrogans on a glass slide (left) and un-spiked urine control (right). Leptospires were fixed and probed with the MAb and green fluorescent conjugated anti-mouse IgM. The reaction was observed under a fluorescence microscope at 1000x magnification.

can detect leptospiral DNA at the early phase of infection[11, 15]. However, molecular methods require sophisticated equipment, trained technicians, and expensive testing materials. As such, these methods would likely not be available in resource-limited settings. Until now, no reliable, affordable and widely available method has been developed that can be used in routine clinical settings at all levels of care. In the acute phase, leptospires are present in the blood and they invade visceral organs[1]. Leptospires and their antigens are present in both blood and urine[1, 17]. The detection of a common specific leptospiral antigen using a specific antibody should be an alternative method for diagnosing leptospirosis[12]. Comparison between blood and urine samples revealed the concentration of antigen and the window period for antigen detection to be lower and narrower in blood than in urine[1]. In this study, a specific MAb isotype (IgMK) was produced. The specificity of the MAb was tested against a broad range of pathogenic and nonpathogenic *Leptospira* serovars, and against 8 other bacterial genera. The 8 bacterial genera (*A. hydrophila, E. coli, K. pneumoniae, Proteus* spp., Ps. *aeruginosa, Salmonella* spp., *Shigella* spp. and *Streptococcus* spp.) were included in the panel antigen because they can cause systemic infection and be detected in blood and urine clinical samples. The MAb and MAb-IFA have to produce no cross reactivity to these bacteria otherwise the false positive result of the MAb-IFA will be obtained. Our MAb was reactive only to antigens prepared from *Leptospira* spp. (both pathogenic and nonpathogenic species), but not reactive to any of the other heterologous antigens that we tested. The immunoblotting pattern appeared as smear bands located at low molecular weight, which is similar to a lipopolysaccharide pattern[18, 19]. In addition, the specific antigenic component reactive to the produced MAb is carbohydrate in nature; since it disappeared after digestion with sodium periodate (Fig. 2). This evidence supported that the specific antigenic component may be a carbohydrate portion of a lipopolysaccharide (LPS). LPS composes of 3 parts, lipid A, core oligosaccharide, and O- antigen (or O polysaccharide)[21]. The composition of the O-antigen varies from serovar to serovar and serogroup to serogroup. Core oligosaccharide commonly contains sugars such as heptose and 3-deoxy-D-manno-oct-2-ulosonic acid (also known as KDO, keto-deoxyoctulosonate). Therefore, a common specific leptospiral antigen might be in the core oligosaccharide part. Most IFA was developed for *Leptospira* antibody detection[22-24]. The MAb-IFA was also developed for *Leptospira* antigen detection. The target antigen is LipL32[25]. The MAb-IFA was able to react with intact or methanol fixed leptospires. However, clinical trial and the limit of *Leptospira* detection were not reported.

Conclusion

A broadly specific IgMκ monoclonal antibody (MAb) was produced by immunization of BALB/cJ mice with secretory antigen (SC) prepared from *L. interrogans* serovar Bratislava serogroup Australis. It reacted to 24 *Leptospira* serovars which are the representative of 19 serogroups but not with other 8 bacterial genera. The evidence seems to indicate that the specific antigen component is a carbohydrate portion of a lipopolysaccharide. Antigen detection by MAb-IFA performed on spiked urine samples revealed that a minimum concentration of 1.5 leptospires/μL or 30 leptospires per 20 uL of applied sample could be detected. The MAb-IFA is a sensitive and low-cost antigen detection assay that can be performed in a routine clinical laboratory. Early and accurate diagnosis of leptospirosis will facilitate improved care and better patient outcomes.

Conflict of interest

All authors declare no personal or professional conflicts of interest, and no financial support from the companies that produce and/or distribute the drugs, devices, or materials described in this report.

Acknowledgement

This study was supported by a grant from the National Science and Technology Development Agency (NSTDA), Pathum Thani, Thailand (grant no. BT - B - 06 - 2E - 14 - 302). (Professor Dr. Suttipant Sarasombath is the principal investigator)

References

1. Haake DA, Levett PN. Leptospirosis in Humans. In: Adler B, editor. *Leptospira* and Leptospirosis. Berlin, Heidelberg: Springer Berlin Heidelberg. 2015; p. 65-97.
2. Bharti AR, Nally JE, Ricaldi JN, *et al.* Leptospirosis: a zoonotic disease of global importance. Lancet Infect Dis 2003; 3: 757-71.
3. Douglin CP, Jordan C, Rock R, Hurley A, Levett PN. Risk factors for severe leptospirosis in the parish of St. Andrew, Barbados. Emerg Infect Dis 1997; 3: 78-80.
4. Adler B, de la Peña Moctezuma A. *Leptospira* and leptospirosis. Vet Microbio. 2010; 140: 287-96.
5. Cesar KR, Romero EC, de Bragança AC, Blanco RM, Abreu PAE, Magaldi AJ. Renal Involvement in Leptospirosis: The Effect of Glycolipoprotein on Renal Water Absorption. PLOS ONE. 2012; 7: e37625.
6. Cerqueira TB, Athanazio DA, Spichler AS, Seguro AC. Renal involvement in leptospirosis--new insights into pathophysiology and treatment. Braz J Infect Dis. 2008; 12: 248-52.

7. Dolhnikoff M, Mauad T, Bethlem EP, Carvalho CR. Pathology and pathophysiology of pulmonary manifestations in leptospirosis. Braz J Infect Dis 2007; 11: 142-8.
8. Peces R. Acute renal failure in severe leptospirosis. Nephrol Dial Transpl 2003; 18: 1235-6.
9. Dupont H, Dupont-Perdrizet D, Perie JL, Zehner-Hansen S, Jarrige B, Daijardin JB. Leptospirosis: prognostic factors associated with mortality. Clin Infect Dis 1997; 25: 720-4.
10. Rajapakse S, Rodrigo C, Handunnetti SM, Fernando SD. Current immunological and molecular tools for leptospirosis: diagnostics, vaccine design, and biomarkers for predicting severity. Ann Clin Microbiol Antimicrob 2015; 14: 2.
11. Musso D, La Scola B. Laboratory diagnosis of leptospirosis: A challenge. J Microbiol Immunol 2013; 46: 245-52.
12. Saengjaruk P, Chaicumpa W, Watt G, *et al.* Diagnosis of human leptospirosis by monoclonal antibody-based antigen detection in urine. J Clin Microbiol 2002; 40: 480-9.
13. Ekpo P, Rungpanich U, Suwancharoen D, Phulsuksombath D, Wuthiekanun V, Suputtamongkol Y. Monoclonal antibody-based latex agglutination for rapid identification of leptospires isolated from patient blood and rat kidney in Thailand. Southeast Asian J Trop Med Public Health 2017; 48: 1210-21.
14. Sarasombath S, Lertmemongkolchai G, Banchuin N. Characterization of monoclonal antibodies to protein antigen of Salmonella typhi. J Clin Microbiol 1988; 26: 508-12.
15. Toyokawa T, Ohnishi M, Koizumi N. Diagnosis of acute leptospirosis. Expert Rev Anti Infect Ther 2011; 9: 111-21.
16. De Abreu Fonseca C, Teixeira de Freitas VL, Caló Romero E, *et al.* Polymerase chain reaction in comparison with serological tests for early diagnosis of human leptospirosis.Trop Med Int Health 2006; 11: 1699-707.
17. Gomes-Solecki M, Santecchia I, Werts C. Animal Models of Leptospirosis: Of Mice and Hamsters. Front Immunol 2017; 8: 58.
18. Vanithamani S, Shanmughapriya S, Narayanan R, *et al.* Lipopolysaccharide Specific Immunochromatography Based Lateral Flow Assay for Serogroup Specific Diagnosis of Leptospirosis in India. PLOS ONE. 2015; 10: e0137130.
19. Priya CG, Bhavani K, Rathinam SR, Muthukkaruppan VR. Identification and evaluation of LPS antigen for serodiagnosis of uveitis associated with leptospirosis. J Med Microbiol 2003; 52: 667-73.
20. Maneewatchararangsri S, Reamtong O, Kalambaheti T, *et al.* Development of enzyme-linked immunosorbent assay for human Leptospirosis serodiagnosis using *Leptospira* secretion antigen. Southeast Asian J Trop Med Public Health 2017; 48: 576-84.

21. Steimle A, Autenrieth IB, Frick JS. Structure and function: Lipid A modifications in commensals and pathogens. Int J Med Microbiol 2016; 306: 290-301.
22. Pradutkanchana S, Pradutkanchana J, Khuntikij P. Detection of IgM specific antibody using indirect immunofluorescent assay for diagnosis of acute leptospirosis. J Med Assoc Thai 2003; 86: 641-6.
23. Appassakij H, Silpapojakul K, Wansit R, Woodtayakorn J. Evaluation of the immunofluorescent antibody test for the diagnosis of human leptospirosis. Am J Trop Med Hyg 1995; 52: 340-3.
24. Koraluru M, Bairy I, Varma M, Vidyasagar S. Diagnostic validation of selected serological tests for detecting scrub typhus. Microbiol Immunol 2015; 59:371-4. doi: 10.1111/1348-0421.12268.
25. Fernandes CP, Seixas FK, Coutinho ML, *et al.* Monoclonal antibodies against LipL32, the major outer membrane protein of pathogenic *Leptospira:* production, characterization, and testing in diagnostic applications. Hybridoma 2007; 26: 35-41