Production and characterization of monoclonal antibodies against leptospira interrogans serovar sanilae

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Abstract

Leptospirosis is a widespread zoonotic disease in tropical areas caused by pathogenic gram – negative spirochetes *Leptospira* spp., which affect both human and animals. The disease is transmitted by contacting urine of the infected animals. Leptospira penetrates through mucosa or open wound skin of infected individuals. Symptoms of leptospirosis are extremely broad such as flu – like illness, red eyes, diarrhea or vomiting. If patients are not diagnosed or treated in time, symptoms can become severe sepsis with multi – organ failure. This study aimed to generate monoclonal antibodies (MAbs) against pathogenic Leptospira spp. that can be used in a development of immunological based assay for early diagnosis of leptospirosis. Mice were immunized with whole cells of fixed *Leptospira interrogans serovar Manilae*. After conventional cell hybridization technique, all MAbs were screened by enzyme – linked immunosorbent assay (ELISA) with sonicated cell lysates of various serovars of leptospira and other bacteria. The results showed that two MAbs obtained in this study bound specifically with *L. interrogans serovar Manilae* and *serovar Pomona* but did not interact with other tested bacteria. The sensitivity indicated by the half maximal effective concentration (EC50) and limit of detection (LOD) by individual MAb was in the range of 4×10^6–7×10^6 cells/ml and 4×10^5–5×10^5 cells/ml, respectively. Both clones of MAbs were isotyped as IgM. Therefore, these preliminary studies indicated that the obtained MAbs can be used for application in an immunological detection of *L. interrogans serovar Manilae*.

Keywords: Monoclonal antibodies, *Leptosipira* spp., ELISA

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1. Introduction

Leptospira is the genus of gram–negative, facultative anaerobic and spirochetes bacteria with an optimum growth temperature at 28–30°C (Faine *et al.,* 1999). The genus is comprised of two species: pathogenic and saprophytic species. However, these species could be subdivided into more than 260 and 60 serovars of pathogenic and saprophytic leptospires, respectively. Classification of these serovar is based on the expression of the surface exposed epitopes of the lipopolysaccharide (LPS) antigen, and the specificity of epitopes depends on their sugar composition and orientation (Adler and Moctezuma, 2010).

Leptospirosis is a widespread zoonosis and is considered as an emerging global public health disease (Vijayachari *et al.,* 2008). This disease is caused by pathogenic *Leptospira* spp. that transmitted by direct contact with the urine of infected animals or contaminated water and soil. The bacteria can enter the body through mucous membranes and opened-wound skin of patients. Although, leptospirosis can be cured easily with antibiotic therapy (Chakraborty *et al.,* 2010), but the clinical presentation of leptospirosis is similar to other febrile illnesses, thus complicating the diagnosis. Symptoms of leptospirosis are high fever, headache, muscle aches, hemorrhage, vomiting, diarrhea, red eyes, jaundice, abdominal pain or a rash. If patients are not treated in time, they may develop meningitis, renal damage, liver failure and respiratory distress (Bolin, 1996). So, early and accurate diagnosis is useful for proper treatment.

There are various methods for diagnosis of leptospirosis, such as isolation of leptospires from patient's sample, dark-field microscopy, polymerase chain reaction (PCR) assays, and immunological assays. Detection of leptospires by isolation from patient's samples requires more than a month and does not suitable for early diagnosis. Direct observation of leptospires by dark-field microscopy is unreliable. And the PCR assays need well-trained specialist for operating high technology equipments. So diagnosis by immunological assays is usually an alternative choice due to its simple, low-cost, high specificity and shorter detection time. Currently, the widely used format is the microscopic agglutination test (MAT). Although, it has an advantage of being specific for serovars, it needs various serovars of leptospires in the detection (Adler and Moctezuma, 2010; Musso and Scola, 2013). Enzyme-linked immunosorbent assay (ELISA) has also been studied. Monoclonal antibodies (MAbs) against *L. interrogans serovar* Pomona were produced. The MAbs reacted not only with *L. interrogans serovar* Pomona but also with serovars Grippotyphosa, Canicola, Icterohaemorrhagiae and Hardjo (Ainsworth *et al.,* 1985). Productions of MAbs against a lipoprotein (LipL32) of the outer membrane of pathogenic Leptospira spp. (Lüdtke *et al.,* 2003) and against leptospiral lipopolysaccharide (Widiyanti *et al.,* 2013) were investigated.

Since, immunological methods are considered to be a suitable method for screening patient's infectious samples and MAbs against other *Leptospira* spp. are still in need. This study aims to produce and characterize monoclonal antibodies specific to *L. interrogans serovar Manilae*, another type of pathogenic *Leptospira* spp.

2. Material and Methods

2.1 Bacterial and antigen preparation

Leptospira interrogans serovar Manilae and *Pomona* were cultured in Ellinghausen – McCullough – Johnson – Harris (EMJH) medium, and incubated at 30°C for 7–10 days while other bacterial strains were cultured in Luria broth (LB) with agitation at 37°C for 24 h. Number of viable cells was counted under a dark-field microscope. All bacteria were harvested by centrifugation at 10,000×g for 10 min at 4°C, washed twice with 0.01 M Phosphate buffer saline (PBS), pH 7.4 and resuspended in PBS. For immunization, bacterial cells were fixed overnight by 4% paraformaldehyde and used as the immunogen. For detection, cells were sonicated at 20 kHz for 2 min and used in ELISA.

2.2 Immunization of mice

Three 8-week-old, female ICR mice were immunized by intraperitoneal (i.p.) injection with 100 µl of fixed suspension (10^7 cells ml^{-1}) of *L. interrogans serovar Manilae* in Freund's complete adjuvant (Sigma) at 1:1 (v/v). Booster injections at two week interval were performed using fixed suspension in Freund's incomplete adjuvant. Mice antiserums were collected by tail bleeding and determined antiserum titer using indirect ELISA. Three days prior to cell fusion, final boost of fixed suspension in normal saline solution was performed.

2.3 Production of monoclonal antibodies

The spleen cells of the immunized mice were removed and fused with NS1 myeloma cells using 50% polyethylene glycol (PEG). The fused cells were cultured in hypoxanthine aminopterin thymidine (HAT) medium containing 20% fetal calf serum at 37°C with 5% CO_2. After 7–10 days, hybridoma culture supernatants from each clone against *L. interrogans serovar Manilae* were screened by ELISA. The positive clones were recloned to obtain monoclone by a limiting dilution method, and stored in liquid nitrogen.

2.4 Screening of hybridoma

Culture supernatants from hybridoma cells were tested for monoclonal antibodies against *Leptospira* spp. using indirect ELISA procedure. Plates were coated with sonicated bacteria samples (L. interrogans serovar Manilae or other bacteria) at a concentration of 1×10^7 cells/ml in a volume of 50 µl per well and incubated at 4°C overnight. Plates were washed with PBS containing 0.05% Tween 20 (PBST) and blocked with 5% skim milk in PBS at 37°C for 1 hr. After washing, hybridoma cell culture supernatants were added and plates were incubated at 37°C for 2 h. Plates were washed and added secondary antibody (horseradish peroxidase-conjugates goat anti mouse IgG; GAMP-HRP) diluted 1:10,000 with PBS, incubated at 37°C for 1 h and washed. Plates were added with substrate solution (3, 3', 5, 5'-tetramethylbenzidine; TMB and H_2O_2 in 205 mM citrate buffer, pH 4.0). After 10 min, the reaction was stopped with 1 M H_2SO_4 and was measured absorption at 450 nm using microtiter plate reader. Then the positive clones were selected by a cut-off value of 0.2.

2.5 Characterization of monoclonal antibodies

2.5.1 Specificity test

Specificity of the MAbs, was determined by ELISA based on the reactivity with *L. interrogans serovar Manilae*. They were assessed for cross-reactivity with other *Leptospira* spp. (*L. interrogans serovar Manilae* and *serovar Pomona*), Gram-negative bacteria (*Enterbacter aerogenes, Enterrococcus faecalis, Escherichia coli, Klebsiella pneumonia, Proteus mirabilis, Pseudomonas aeruginosa,Serratia marcescens*) and Gram-positive bacteria (*Staphylococcus epidermidis*) that found in urinary tract infection at a concentration of 1×10^7 cells/ml.

2.5.2 Sensitivity test

Sensitivity test was determined by an indirect ELISA. Plates were coated with 50 µl per well of two-fold serial dilutions of sonicated *L. interrogans serovar Manilae* from 3×10^7 to 1×10^5 cells/ml dilute in PBS and incubated overnight at 4°C.

The sensitivity of the obtained MAbs was justified based on the half maximal effective concentration (EC50) which was defined as the concentration at which 50% B/B_0 was obtained, where B and B_0 is the average of absorbance obtained from indirect ELISA with or without *L. interrogans serovar Manile*, respectively (Oaew *et al.,* 2012).

$$\%\frac{B}{B_0} = \left[B_0 + \frac{B\text{-}B_0}{1+10^{\log EC50-x}}\right] \times 100$$

The limit of detection (LOD) was determined by titration with serial dilution of *L. interrogans serovar Manile* and calculated by subtracting the concentration at B_0 values with three times of its standard deviation (SD) values, LOD = [3SD].

2.5.3 Isotype determination

Isotype of MAbs was determined for class and subclass using Sigma-Aldrich's mouse monoclonal antibody isotyping kit based on a sandwich ELISA. Plates were coated with isotyping specific antibodies: IgG1, IgG2a, IgG2b, IgG3, IgM and IgA, incubated at 37°C for 1h. After washing, culture supernatant was added and plates were incubated at 37°C for 1 h. After washing, HRP goat anti-mouse IgG (Fab specific) was added and incubated at 37°C for 30 min. After another washing, the assay was performed as described previously.

3. Results and Discussion

3.1 Antiserum titer

After immunization, antiserum titer of blood samples of immunized mice was determined by the indirect ELISA. The result shown in Figure 1 suggested that the end point titers of the two immunized mice were 1:8,000 and 1:16,000 respectively. Immune-reactivity of the pre-immunized mice serum was used as the negative control.

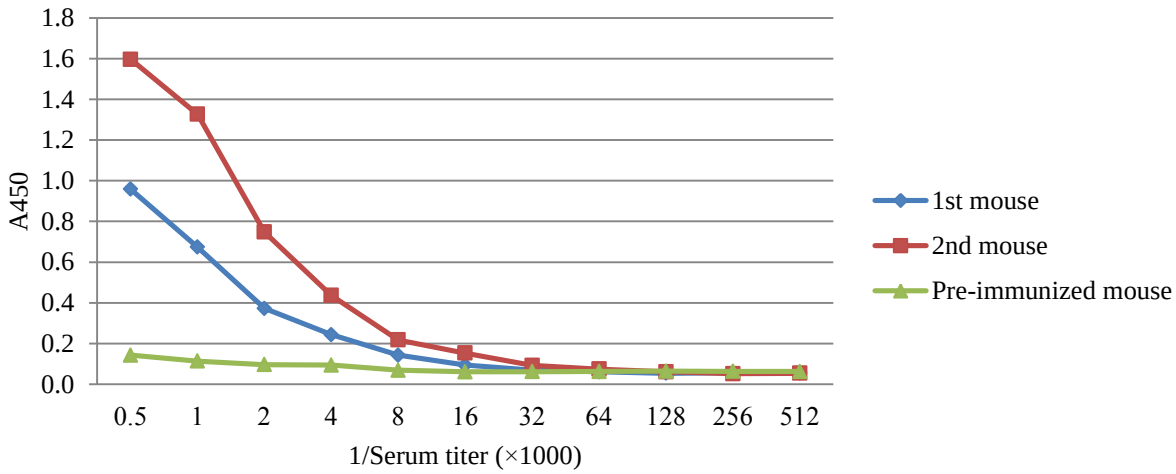

Figure 1 Antiserum titers of immunized and pre-immunized ICR mice against *L. interrogans serovar Manilae* were determined by indirect ELISA.

3.2 Efficiency of fusion

During the somatic cell fusion to produce hybridoma cell, the fusion efficiency (the amount of wells that showing cell multiplication) was 100% and 70% in the first fusion and second fusion, respectively.

3.3 Hybridoma cell selection

The presence of antibodies against *L. interrogans serovar Manilae* hybridoma supernatant was analyzed by the indirect ELISA. The result showed that 10% of hybridomas from the first trial gave positive result while 7% of the second fusion was positive. Using indirect ELISA, thirteen clones of the positive wells from both fusions gave positive result indicating the ability to bind with *L. interrgans serovar Manilae*. All clones were subcloned by limiting dilution until 1 cell per well or monoclone was obtained. Finally, the stable hybridoma cells were selected and MAbs were produced for further characterization.

3.4 Characterization of monoclonal antibody

3.4.1 Specificity

Out of thirteen clones, three stable monoclones 5/2EF1, 5/7CF2 and 10/1GF2 were obtained. Their specificities were shown in Figure 2. The clones were divided into two groups depending on their specificities by indirect ELISA. MAbs 5/7CF2 reacted with *L. interrogans serovar Manilae* and other tested bacterial species except for E. coli and S. marcescens. While MAb 5/2EF1 and 10/1GF2 were specific to *L. interrogans serovar Manilae* and did not cross-reacted with other tested bacterial species. These findings indicated that MAb 5/2EF1 and 10/1GF2 were suitable for further use in diagnosis development. Therefore, they were selected for sensitivity and isotype analysis.

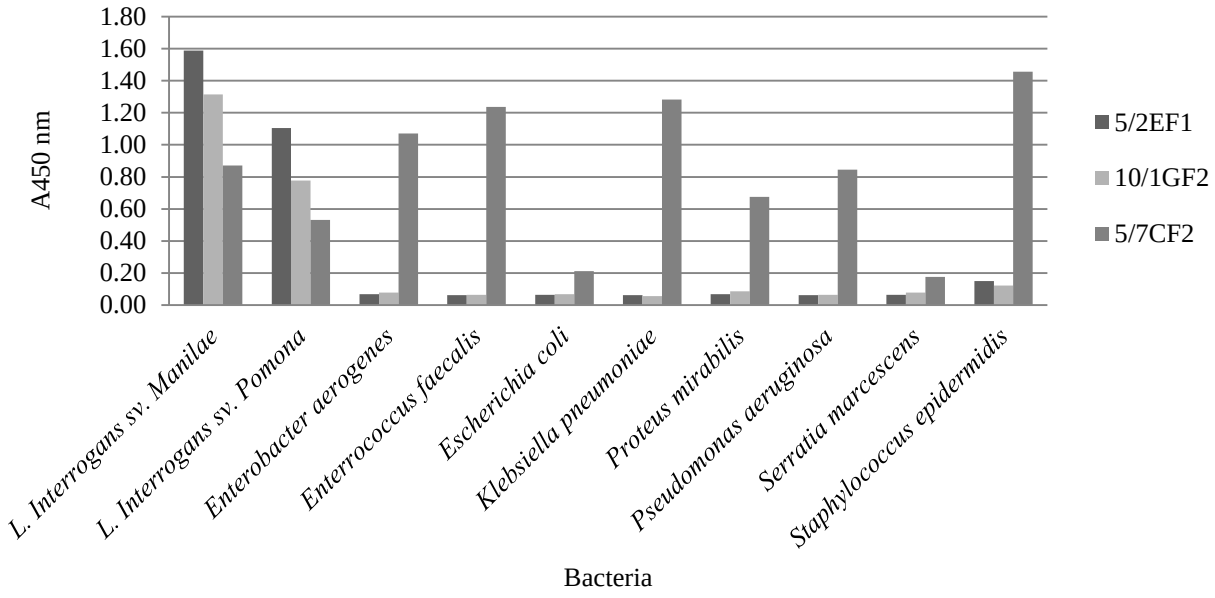

Figure 2 Specificity of MAbs against L. interrogans sv. Manilae and other bacteria

3.4.2 Sensitivity

The sensitivity of MAb 5/2EF1 and 10/1GF2 was determined by the indirect ELISA in terms of EC50 and LOD values. The EC50 values were quantified in the range of 4.34×10^6 - 7.45×10^6 cells/ml and the LODs were between 4.19×10^5 and 5.84×10^5 cells/ml.

3.4.3 Isotype

Isotyping of MAb 5/2EF1 and 10/1GF2 was identified by Sigma-Aldrich's mouse monoclonal antibody isotyping kit. The absorbance values from the ELISA were shown in Table 1. The results indicated that the isotype of the two MAbs was IgM.

Table 1 The absorbance values at 450 nm in ELISA-based isotyping determination

Clones	A450 nm					
(Group 2)	IgG1	IgG2a	IgG2b	IgG3	IgM	IgA
5/2EF1	0.069	0.135	0.075	0.079	0.713	0.078
10/1GF2	0.071	0.153	0.085	0.523	1.450	0.115

4. Conclusion

Using conventional hybridoma cell fusion, two MAbs (5/2EF1 and 10/1GF2) that showed ability to specifically bind with *L. interrogans serovar Manilae* were obtained. Both antibodies were identified as IgM with the EC50 and the LODs values in the range of 4.34×10^6 –7.45×10^6 cells/ml and 4.19×10^5 and 5.84×10^5 cells/ml, respectively.

References

Adler, B., Moctezuma, A.P. 2010. Leptospira and leptospirosis. Veterinary Microbiology. 140, 287–296.

Ainsworth, A.J., Lester, T.L., Capley, G. 1985. Monoclonal antibodies to Leptospira interrogans serovar Pomona. Canadian Journal of Comparative Medicine. 49, 202–204.

Bolin, C. 1996. Diagnosis of leptospirosis: a reemerging disease of companion animals. Seminars in Veterinary Medicine and Surgery (Small Animals). 11, 166–171.

Chakraborty, A., Miyahara, S., Villanueva, Y.A.M.S., Gloriani, G.N., Yoshida, S. 2010. In vitro sensitivity and resistance of 46 Leptospira strains isolated from rats in the Philippines to 14 antimicrobial agents. Antimicrobial Agents and Chemotherapy. 12, 5403–5405.

Faine, S., Adler, B., Bolin, C., Perolat, P. 1999. Leptospira and Leptospirosis. 2nd Edition. Medisci Press, Melbourne, Australia.

Lüdtke, C.B., Coutinho, M.L., Jouglard, S.D.D., Moreira, C.N., Fernandes, C.H.P., Brod, C.S., Haake, D.A., Ko, A.I., Dellagostin, O.A., Aleixo, J.A.G. 2003. Monoclonal antibodies against an outer membrane protein from pathogenic Leptospira. Brazilian Journal of Microbiology. 34, 1–4.

Musso, B., Scola, B.L. 2013. Laboratory diagnosis of leptospirosis: A challenge. Journal of Microbiology, Immunology and Infection. 46, 245–252.

Oaew, S., Charlermroj, R., Pattarakankul, T., Karoonuthaisiri, N. 2012. Gold nanoparticles/ horseradish peroxidase encapsulated polyelectrolyte nanocapsule for signal amplification in Listeria monocytogenes detection. Biosensors and Bioelectronics. 34, 238–243.

Vijayachari, P., Sugunan, A.P., Shriram, A.N. 2008. Leptospirosis: an emerging global public health problem. Journal of Bioscience. 33, 557–569.

Widiyanti, D., Koizumi, N., Fukui, T., Muslich, L.T., Segawa, T., Villanueva, S.Y.A.M., Saito, M., Masuzawa, T., Gloriani, N.G., Yoshida, S. 2013. Development of Immunochromatography – Based Methods for Detection of Leptospiral Lipopolysaccharide Antigen in Urine. Clinical and Vaccine Immunology. 20, 683–690.