The Role of Cytokine Response Signatures in the Pathogenesis of Leptospirosis Associated Acute Kidney Injury

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บทบาทของไซโตคายน์ในการเกิดภาวะไตวายเฉียบพลันจากการติดเชื้อเลปโตสไปโรซิส

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ณัฐชัย ศรีสวัสดิ์ : บทบาทของไซโตคายน์ในการเกิดภาวะไตวายเฉียบพลันจากการติดเชื้อเลปโตสไปโรซิส . (The Role of Cytokine Response Signatures in the Pathogenesis of Leptospirosis Associated Acute Kidney Injury) อ.ที่ปรึกษาหลัก : สมชาย เอี่ยมอ่อง

ความเป็นมา ภาวะไตวายเฉียบพลัน เป็นหนึ่งในภาวะแทรกซ้อนที่รุนแรงของโรคเลปโตสไปโรสิส ซึ่งเป็นโรคติดต่อจากสัตว์สู่คนที่สำคัญในเขตร้อน การมีปฏิสัมพันธ์ระหว่าง เชื้อโรคเลปโตสไปราและผู้ป่วย เป็นหนึ่งในปัจจัยสำคัญที่มีโอกาสก่อให้เกิดภาวะไตวายเฉียบพลันในโรคเลปโตสไปโรสิสการศึกษานี้มีวัตถุประสงค์เพื่อศึกษาความสัมพันธ์ของระดับไซโตไคยน์ ปริมาณเชื้อเลปโตสไปรา ปริมาณแอนติบอดีต่อต้านเชื้อเลปโตสไปรา ต่อการเกิดภาวะไตวายเฉียบพลันในโรคเลปโตสไปโรสิส วิธีการศึกษา การศึกษานี้ใช้รูปแบบการศึกษาแบบไปข้างหน้าหลายสถาบัน โดยมี 2 ช่วงระยะเวลา ระยะแรก ศึกษาตั้งแต่สิงหาคม พ.ศ.2555 ถึงพฤศจิกายน พ.ศ. 2557 ระยะที่สอง ทำการศึกษาตั้งแต่เดือนกุมภาพันธ์ พ.ศ.2559 ถึงกรกฎาคม พ.ศ. 2560 ทำการเก็บตัวอย่างเลือดในวันแรกและวันที่ 7 การตรวจเพื่อยืนยันการวินิจฉัยโรคเลปโตสไปโรสิส ใช้เทคนิคมาตรฐาน 3 วิธี (การทดสอบไมโครสโคปิกแอกกลูติเนชั่น, การเพาะเชื้อโดยตรง และเทคนิคปฏิกริยาลูกโซ่พอลิเมอเรส) ใช้เกณฑ์มาตรฐานสำหรับการวินิจฉัยภาวะไตวายเฉียบพลัน ผลการศึกษา จากผู้ป่วย 206 รายที่ได้รับการคัดเลือก 113 รายเป็นผู้ป่วยโรคเลปโตสไปโรซิสที่ยืนยัน พบว่าร้อยละ 37 มีภาวะไตวายเฉียบพลัน ค่ามัธยฐานของระดับ MCP-1 และ TNF-a ในวันแรกของผู้ป่วยที่มีภาวะไตวายเฉียบพลัน มีค่าสูงกว่าผู้ป่วยที่ไม่มีภาวะภาวะไตวายเฉียบพลัน อย่างมีนัยสำคัญ [309.3vs138.8 พิโคกรัมต่อมิลลิลิตร, P = 0.003] และ [492.3 เทียบกับ 77.5 พิโคกรัมต่อมิลลิลิตร, P = 0.003] ตามลำดับ โดยมีค่าพื้นที่ใต้กราฟในการทำนายการเกิดภาวะไตวายเฉียบพลัน เท่ากับ 0.67 และ 0.67 ตามลำดับ จากการวิเคราะห์แบบหลายตัวแปร มีเพียงระดับ IL-6 ที่ต่ำเท่านั้นที่แสดงให้เห็นถึงความสัมพันธ์กับภาวะไตวายเฉียบพลัน นอกจากนี้ปริมาณเชื้อเลปโตสไปโรที่สูงในกระแสเลือดและปริมาณแอนติบอดีต่อเชื้อเลปโตสไปราที่สูง มีความเกี่ยวข้องกับภาวะไตวายเฉียบพลันที่รุนแรง สรุป จากการศึกษานี้พบว่าระดับ เอมซีพี วัน และ ทีเอนเอฟ อัลฟ่า มีความสัมพันธ์ต่อการเกิด ภาวะไตวายเฉียบพลัน ในผู้ป่วยโรคเลปโตสไปโรสิส อย่างไรก็ตามในรูปแบบการวิเคราะห์ข้อมูลแบบหลายตัวแปร มีเพียงระดับ อินเตอร์ลิวคิน ซิก ที่ต่ำเท่านั้นที่เกี่ยวข้องกับภาวะไตวายเฉียบพลัน นอกจากนี้ปริมาณเชื้อเลปโตสไปโรที่สูงในกระแสเลือดและระดับแอนติบอดีเลปโตสไปราที่สูงมีความสัมพันธ์ต่อการเกิดภาวะไตวายเฉียบพลันที่รุนแรง

สาขาวิชา ชีวเวชศาสตร์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2561 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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Nattachai Srisawat : The Role of Cytokine Response Signatures in the Pathogenesis of Leptospirosis Associated Acute Kidney Injury. Advisor: Prof. Somchai Eiam-Ong, M.D.

Background: Acute Kidney Injury (AKI) *is* one of the most serious complications of leptospirosis, an important zoonosis in the tropics. Host pathogen interaction is one of the potential key factor in development of leptospirosis associated AKI. In this multicenter study, we aimed to study the association of cytokines, leptospiral burden, and anti-leptospira antibody and AKI in leptospirosis. *Method:* In the first cohort, patients who presented with clinical suspiciousness of leptospirosis were prospectively enrolled in 9 centers from August 2012 to November 2014. In the second cohort, we prospectively recruited patients from 15 centers from February 2016 to July 2017. The first day of enrollment was the first day of clinical suspicious leptospirosis. Blood samples were serially collected on the first day and day 7 after enrollment. We used three standard techniques (microscopic agglutination test, direct culture, and PCR technique) to confirm the diagnosis of leptospirosis. KDIGO criteria were used for AKI diagnosis. *Results:* Of the 206 recruited cases, 113 cases were leptospirosis confirmed cases. Thirty seven percent developed AKI. Median MCP-1 and TNF-a on the first day in those developing AKI were significantly higher than in patients not developing AKI [309.3vs138.8 pg/mL, P = 0.003], and [492.3 vs 77.5 ng/ml, P = 0.003], respectively. MCP-1 and TNF-a levels associated with AKI had AUC-ROC of 0.67, and 0.67, respectively. Only low IL-6 showed strong association with AKI by a covariate-adjusted model. High leptospiral burden and high anti-leptospira antibody associated with severe AKI.*Conclusion:* From this multicenter study, MCP-1, TNF- a appears to be useful markers for detecting AKI in leptospirosis patients. In the adjusted model for severity, only low IL-6 is associated with AKI. Our data suggest that high leptospiremia and high anti-leptospira antibody correlate with the severity of AKI.

Field of Study:	Biomedical Sciences	Student's Signature .....
Academic Year:	2018	Advisor's Signature .....

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Nattachai Srisawat

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1.1 Background and rationale

Leptospirosis is an important zoonosis especially in the tropics. However, with the impact of world globalization, there are also reports of this disease as sporadic cases in developed countries. A recent report has shown the annual incidence of leptospirosis in Thailand was 48.9 per million population, thus ranking 7th in the world in terms of incidence.

Acute Kidney Injury (AKI) is one of the most serious complication of leptospirosis. The incidence of AKI in leptospirosis by using the RIFLE AKI criteria was up to 84%. This is higher than the average AKI incidence in the Intensive Care Unit (ICU) and more than twice as high as seen in patients with community acquired pneumonia in the US. In this specific setting the kidney is injured by direct effects (direct invasion of the organism) and by indirect effects such as dehydration, rhabdomyolysis, and hemorrhagic shock.

Cytokines are known to play a major role in the pathogenesis of severe sepsis/septic shock. In severe leptospirosis, immune response to leptospira seem to play a major role. However, the role of immune response in pathogenesis of AKI in leptospirosis is still unknown. Benefits from study cytokine response in leptospirosis associated AKI would include better understand pathogenesis of AKI in leptospirosis patients, identify target intervention, and predict leptospirosis associated AKI outcome.

We plan conduct a prospective observational study to measure the cytokines in plasma of hospitalized patients suspected of having leptospirosis. Using the KDIGO criteria to classify AKI status first, we analyzed baseline characteristics between AKI patients and non AKI patients. Second, we compared the differences in plasma cytokines concentration by AKI and renal recovery status. Third, we examined whether plasma cytokines level associated with AKI and predicted renal non-recovery in a multivariable model adjusting for clinical parameters.

1.2. Research questions

1.2.1 Do the inflammatory cytokines play role in the pathogenesis of leptospirosis AKI?

1.2.2 Do the inflammatory cytokines can predict the recovery phase of leptospirosis AKI?

1.3. Objectives

1.3.1 Primary objective

- To study the role of inflammatory cytokines in pathogenesis of leptospirosis associated AKI

1.3.2 Secondary objective

- To study the role of leptospirosis load and AKI
- To study the role of leptospira species and AKI
- To study the role of inflammatory cytokines in prediction the recovery phase of leptospirosis associated AKI

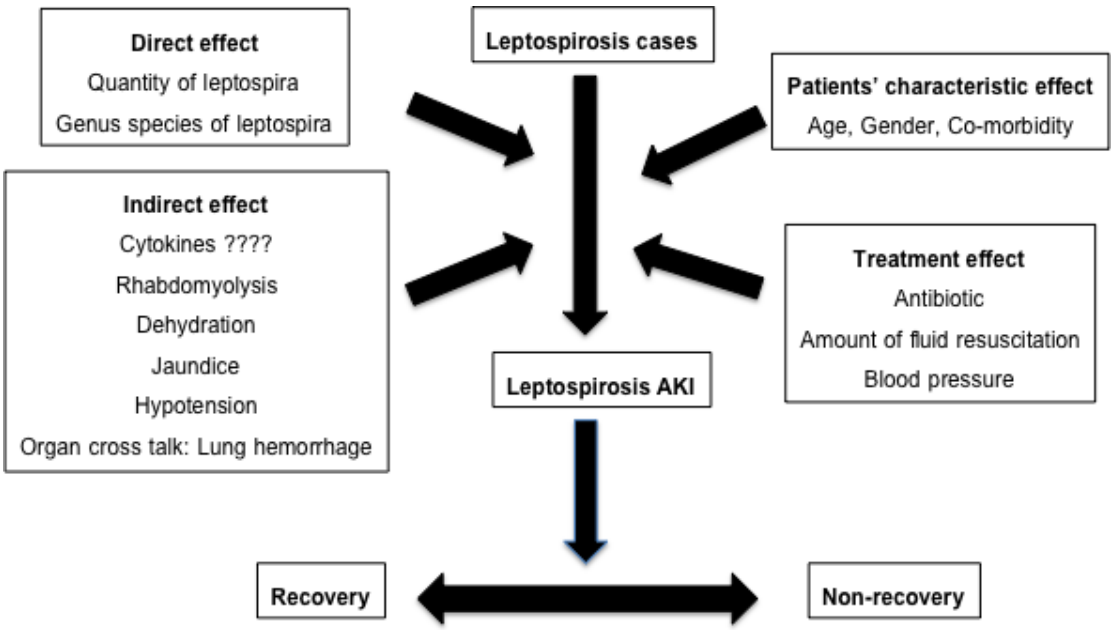
1.4. Hypothesis

- High level of Inflammatory cytokines associated with the high incidence of leptospirosis associated AKI
- High leptospiral load associated with leptospirosis associated AKI
- Specific leptospirosis species associated with leptospirosis associated AKI
- High level of Inflammatory cytokines associated with the renal non recovery of leptosopirosis associated AKI

1.5. Research design

Multicenter, prospective, observational study

1.6. Conceptual frameworks



CHAPTER 2: LITERATURE REVIEW

Leptospirosis is one of the main cause of tropical infections causing acute kidney injury (AKI). These infections commonly relate to the low socioeconomic status and humid climate. Leptospires modified their membrane transport for cell volume regulation and acid base control to survive in the various environments of the tropics. Kidney is one of the main target due to highly vascular blood supply, low rate of leptospires clearance, and suitable acid-base environment. The unique clinical characteristics of leptospirosis associated AKI is non-oliguria, and hypokalemia. The mechanism of leptospirosis associated AKI can be direct or indirect effect. Leptospiral bacteria invade renal interstitium by attachment the part of outer membrane protein to renal tubular epithelium. The indirect effect mostly result from dehydration, rhabdomyolysis, hemoglobinuria, and hyperbilirubinemia as the part of multiple organ dysfunction syndrome. Prompt antibiotic treatment, adequate hemodynamic resuscitation, and early renal support are the key success in the treatment of leptospirosis associated AKI. The concept of the transition from AKI to chronic kidney disease has been recently proposed in the field of nephrology. Unfortunately, the long term renal outcome of these specific diseases is still lacking.

2.1 Overview of leptospirosis

Leptospirosis is an important zoonosis especially in tropical areas. The disease has global impact with an estimated 1.03 million cases worldwide causing 58,900 deaths annually (1). A recent report has shown that the endemic area of leptospirosis include the Caribbean and Central and South America, as well as in Southeast Asia and Oceania. There are sporadic reports in developed countries (2). Classic description of disease starts from definition, epidemiology, pathogenesis, to management and prevention. Ideally, prevention is most important of all. A great deal of investigation has covered nature of leptospires, pathophysiology organ pathology and clinical manifestation.

How leptospires survive in the environment?

Leptpspires are obligate aerobes spirochetes, about 0.1 um in diameter by 6-20 um in length. It comprises the genus Leptospira, which belongs to the family Leptospiraceae, order Spirochaetales. Leptospires have distinctive hooked ends, with two periplasmic flagella and are responsible for motility. Leptospires have a typical double membrane structure in which the cytoplasmic membrane and peptidoglycan cell wall are closely associated and are overlaid by an outer membrane (3). Within the outer membrane, the lipopolysaccharide (LPS) constitutes the main antigen for Leptospira. It is structurally and immunologically similar to LPS from Gram negative organisms. The optimum temperature for the growth of leptospires is 28-30^{o}C (4).

It is fascinating how leptospires inhabiting water and wet soil react to osmolality and temperature changes in the environment for survival. Leptospires suffer from the heavy rain when flooded rain water is clear with low osmolarity and acid pH. Leptospiral cell membrane has been the subject of great interest with detailed study (5). Membrane transport of molecules involved in cell volume regulation is not well defined, but perhaps is close to that of animal (6, 7) With clear water, leptospires expose fully to ultraviolet light that can cause injury. Rain water with almost zero solute is hypoosmotic causing cell swelling. In addition to curling to decrease the surface area, leptospires would react by closing all Na channels and eliminate K which is an important intracellular solute by K-CI cotransport. Loss of K and Cl succeeds in volume regulation, but can lead to cell death. Elimination of H^{+} from cells is hampered due to acid rain pH. NHE is down regulated. At any rate, the rain helps leptospires in dissemination to the host. They must enter the host as fast as

possible targeting in blood stream with favorable alkaline pH. Penetration into the skin by cut or abrasion is the common route. With mobility in cork screw fashion, they can reach blood stream and multiply.

Direct contact of domestic and wild animal species with infected urine, or contaminated water sources, facilitates transmission of leptospirosis, because leptospires can penetrate breaches of the skin or mucosal surfaces, e.g., the conjunctiva. Early studies of mice, rats, and guinea pigs showed that uninjured skin and nasal mucosal membranes are barriers to Leptospira infection (8).

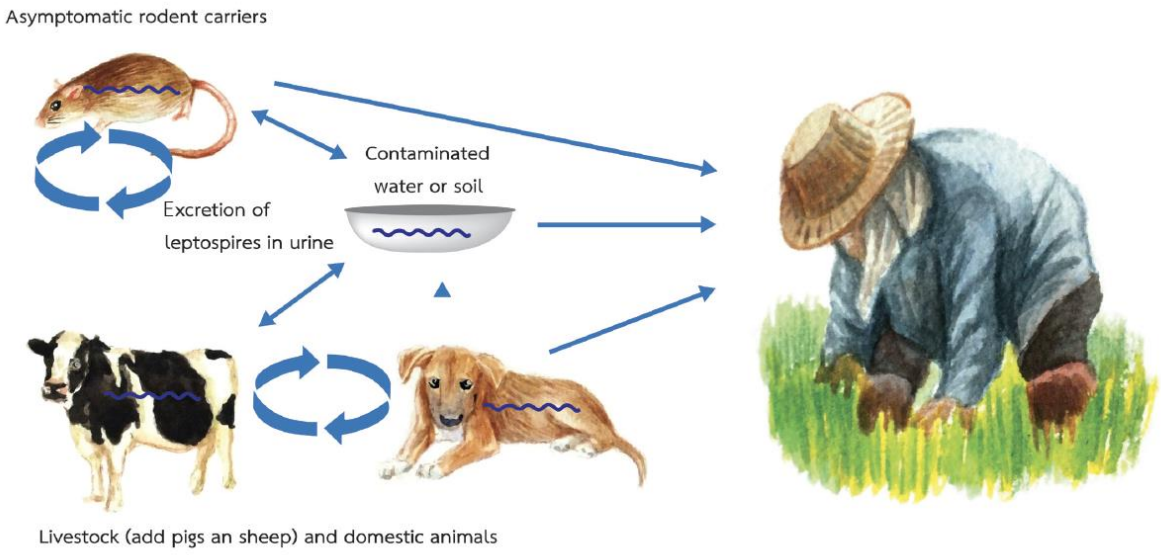


Figure 1. The transmission route among human, animal, and environment.

2.2 Kidney as a tropism organ for leptospires

With mobility in cork screw fashion, leptospires can reach blood stream and multiply. They face several defense mechanisms from the host through the interaction between the outer membrane and toll like receptor (TLR) Mobility of leptospires is an important asset for invasion. They enter the kidney cells through interstitium to survive from immune mechanism of the host. Epididymis, choroid plexus and lung which deal with acid-base and transport mechanism are also involved in leptospiral infection (9, 10). The kidney is a primary target of leptospires during both acute and chronic infection, where conditions in the renal tubules favor leptospires survival. Even though, leptospires multiply and migrate randomly through

all body tissues, there are three main predisposing factors which make kidney the vulnerable organ for leptospiral infection. First, kidney is the highly vascular organ and receive the blood supply from 25% of cardiac output (11). Secondly, data from experimental infection in rat (rattus norvegicus) showed that there was rapid clearance of leptospires in almost of the tissue after extensive dissemination to all tissue, except the kidney, especially proximal convoluted tubule. The clearance from most tissue is likely facilitated by circulating anti-leptospiral immunoglobulin (IgM and IgG)(12). Thirdly, among animal cells, the kidney cells well regulate intracellular osmolality and pH suitable for leptospire survival, and thus are important target (13, 14).

However, to prove the concept of kidney tropism of leptospirosis, there is still missing the pieces of information which need to be provided. First, experimental evidence to support the hypothesis that the kidney is an immune privileged site. Secondly, we need to determine if IgG present in the urine of infected rats is specific for leptospira and whether it reacts with leptospira in the renal tubules in vivo. Thirdly, studying the correlation between the presence of leptospires specific antibody in the urine and the absence of complement is essential and identification of leptospira proteins that which are involved with immune evasion by interfering with the host complement activation system. Fifthly, the effects of environmental factors, such as nutrition, diet, habitat on renal colonization of leptospires during natural infection and how leptospira host invasion in nature compares with leptospira host invasion during experimental infection need to be determined. Finally, it is important to establish leptospira biofilm formation in renal colonization and immune evasion(15).

2.3 Mechanism of leptospirosis causing kidney injury

The mechanism of leptospires causing AKI can be divided into direct kidney damage (acute interstitial nephritis) and indirect damage

Direct damage (acute interstitial nephritis)

Study in the past, showed the evidence of higher leptospiral load in kidney than other organs. Leptospires gain access to the kidney by hematogenous route, causing initially glomerular injury. This demonstrated by the evidence of glomerular congestion, and mild mesangial proliferation 3 hours after inoculation of leptospires in hamster model. Interstitial changes occurred after they reached peritubular

capillaries. Leptospires migrated to the interstitium, renal tubular epithelium, and tubular lumen producing structural damage. Of note, interstitial changes always preceded tubular changes, and both continued on after glomerular changes had stopped (16). Leptospires in the renal tissue triggers a process of acute interstitial nephritis, the key mechanism of AKI in leptospirosis (17).

The outer membrane of leptospires contains antigenic components including lipoproteins, lipopolysaccharides and peptidoglycans, endotoxins that can account for kidney injury, leading to tubular dysfunction and inflammation. Several outer membrane proteins (OMPs) such as lipL32, Loa22, LipL41, Lig family, LipL36, LipL21, and LipL46 of pathogenic species have been identified at the proximal tubule and interstitium of infected animals.(5) Among them, LipL32 is one of the most important OMPs (18). The LipL32 will bind to toll-like receptor 2 (TLR2), not TLR-4, leading to activation NF-kβ. This will stimulate the production of pro-inflammatory cytokines and chemokines such as tumor necrosis factor (TNF-α), inducible nitric oxide, monocyte chemotactic protein-1, T cells (RANTES), and CXCL2/MIP-2 for recruiting inflammatory cells (19, 20). TNF-α is one of the most investigated cytokine in leptospirosis. It is an inflammatory cytokine produced following to TLR-2 stimulation (21). (Figure 2,3)

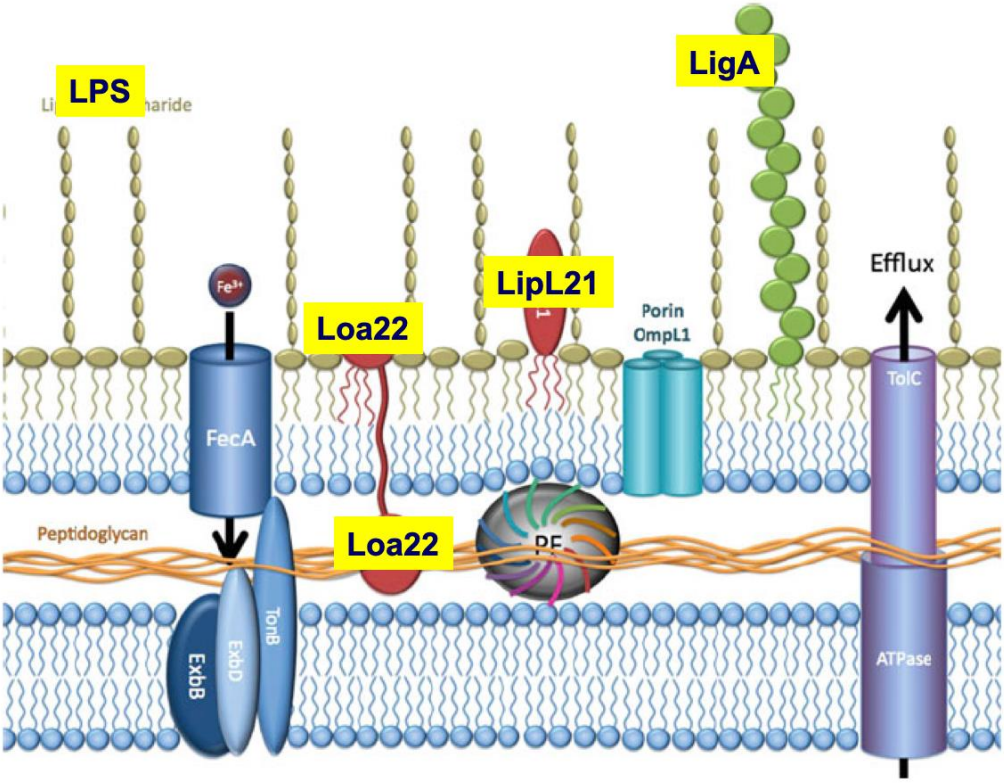


Figure 2. Structure of the outer membrane of leptospires which composed of lipopolysaccharide (LPS), Lipoprotein such as LipL21, Loa21, LigA, and transmembrane protein such as FecA, OmpL1, TolC, and Loa22

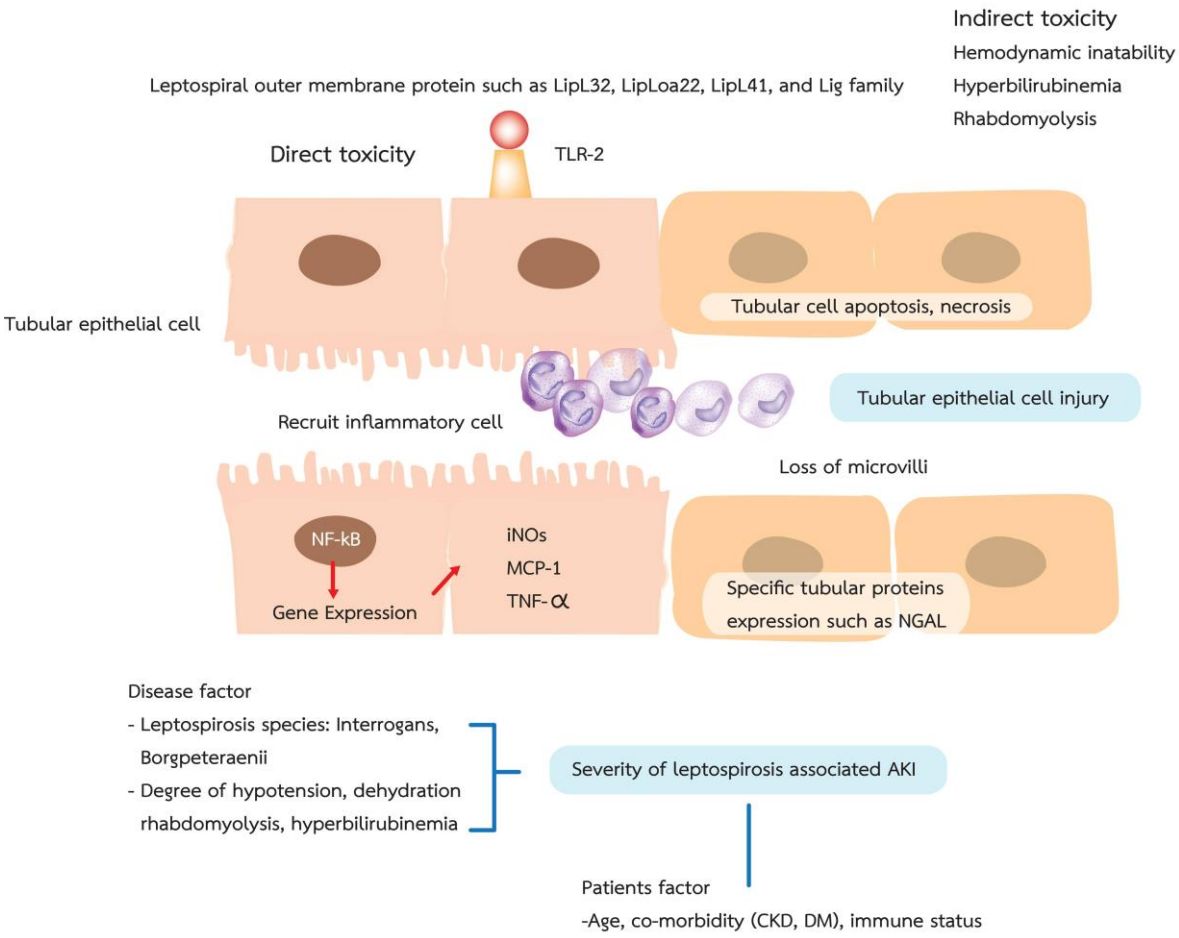


Figure 3. Pathogenesis of leptospirosis associated acute kidney injury (AKI) and factors associated with severity of leptospirosis associated AKI. Leptospires migrated to the renal tubulointerstitium. They produce structural damage by using the outer membrane protein binding to toll like receptor 2 (TLR-2). This will lead to activate NF-kβ and stimulate the production of pro-inflammtory cytokines and chemokines.
iNOS – inducible nitric oxide synthase, OMP– outer membrane protein, MCP -1 Monocyte chemotactic protein-1, TNF- α- tumor necrosis factor- α.

Role of leptospiral load

The role of quantity of leptospira and severity of leptospirosis was first studied by Truccolo J in 2001 (22). Twelve acute leptospirosis patients were recruited **w** ith the mean ages of 36 years; nine were male and three were female. Twenty-seven samples, including 23 blood specimens and four urines, were collected for testing. Interestingly, three patients who died showed the highest number of leptospira in the blood with the minimum number of leptospires at least 10^4/ml of blood. The authors

proposed this number as the critical thereshold for severe leptospirosis. Subsequent study by Agampodi S et al in 381 patients who presented with fever in Sri Lanka. Fifty eight cases were found qPCR positive. The bacterial load in serum ranged from 10^2 to 10^6 Leptospires/mL. Median leptospiral load for patients with mild severity (N=40), with kidney failure (N=8), with myocarditis (N=6), and with multi-organ failure patients (N=4) were 8616, 11007, 36100, and 15882 Leptospira/mL, respectively. However, there was not statistically different among group, (P = 0.59) (23).

Another study from Tubiana et al conducted a retrospective case-control study of inpatients with laboratory-confirmed leptospirosis who were admitted to two public hospitals in New Caledonia between 2008–2011.Controls were defined as patients hospitalized for milder leptospirosis. Risk and prognostic factors were identified by multivariate logistic regression. Among the 176 patients enrolled in the study, 71 had criteria of severity including 10 deaths. Leptospiremia more than 1000 leptospires/mL was associated with mortality (OR = 4.31 [CI 1.17–15.92]) (24).

Indirect kidney damage

In general, hemodynamic changes in leptospirosis is similar to sepsis with ischemic type of AKI depending on the severity of disease. Siriwanij et al reported three patterns of hemodynamic changes during severe leptospirosis. The first which was the most common pattern was decreased systemic vascular resistance (SVR) and mean arterial pressure, increased cardiac index (CI), but normal pulmonary vascular resistance (PVR) and pulmonary capillary wedge pressure (PCWP). The second pattern which was found in patients with pulmonary complication was normal SVR and CI, increased PVR. The last pattern in the setting of hyperbilirubinemia was normal or slightly increased SVR. CI and MAP were decrease while PVR and PCWP were unchanged (25). Secondly, hyperbilirubinemia is common in severe leptospirosis. Sitprija et al demonstrated the toxicity of hyperbilirubinemia in the model of obstructive jaundice from cholangiocarcinoma. Bilirubin levels higher than 26 mg/dL impaired glomerular filtration rate (GFR) and ability to concentrate urine (26). Thirdly, rhabdomyolysis is another common indirect cause of kidney injury. The incidence of rhabdomyolysis in leptospirosis is up to 60%. Rhabdomyolysis causes

kidney injury via renal vasoconstriction, tubular obstruction, and direct toxicity (24-26). Figure 3 demonstrates the pathogenesis of leptospirosis associated AKI.

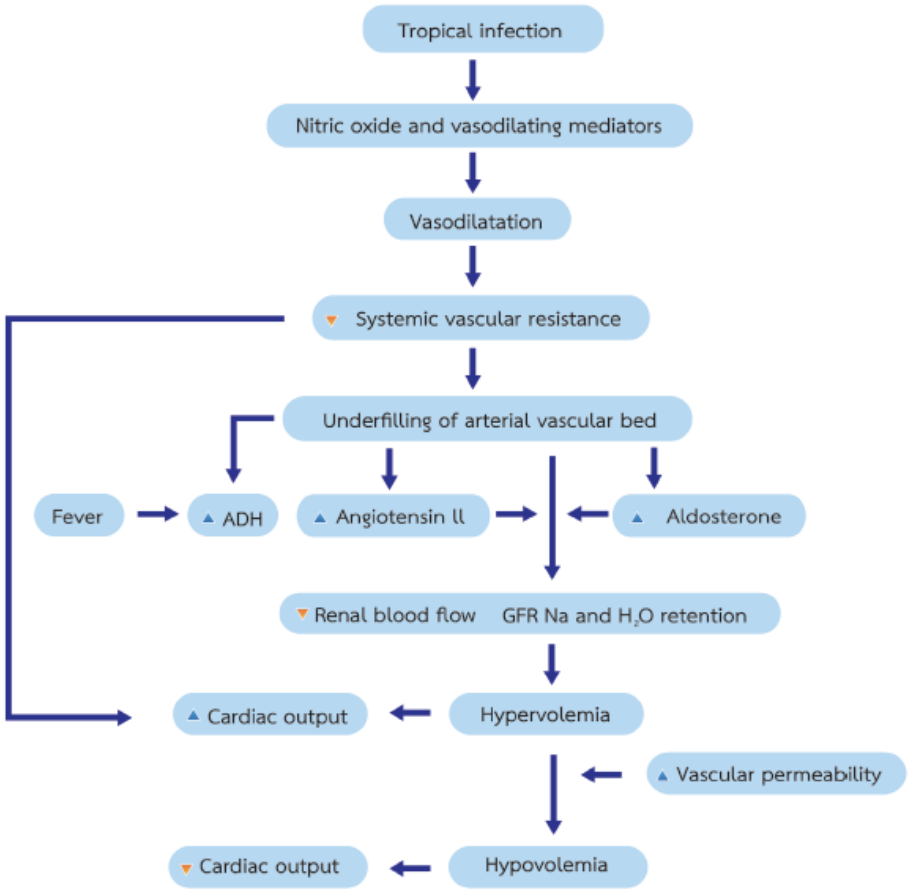


Figure 4. The hemodynamic alteration after leptospirosis infection

Role of cytokines

Tumor Necrosis Factors (TNF-α)

Tumor necrosis factor-alpha (TNF-α) is considered as one of the widely studied cytokine and is a prominent mediator for severe leptospirosis. TNF-α is a pro-inflammatory cytokine produced by monocytes, macrophages and resident renal cells that plays crucial roles in various biological events including vasodilatation, tissue development and death, cell differentiation and immune-mediated diseases (27).

Estavoyer et al. was the first person who reported the case series of the role of TNF-α in leptospirosis. The investigator found that high level of TNF-α was associated with severe non-fatal leptospirosis (28). Following study from Tajiki et al also demonstrated that TNF-α expression was associated with liver, kidney and lungs

involvement as well as bleeding. In addition, high level of TNF-α was associated with higher mortality seen in patients suffering from severe leptospirosis (29).Similar findings from Kyriakidis et al also showed that high level of TNF-α was related with severe pulmonary hemorrhage (30). This hemorrhagic condition could be due to secretion of hemolysins by leptospires which induces the production of TNF-α. Subsequently, TNF- α will react on endothelium and macrophages, causing vascular inflammation, fluid leakage and ultimately leads to severe pulmonary hemorrhages (31, 32).However, in contrast to the above findings, Reis et al conducted prospectively, enrolled, case control study to compare cytokine profiles in patients with mild (defined as outpatient cases) and severe leptospirosis (defined as hospitalized cases). Of one hundred seventy-two patients were recruited, 23 patients were mild disease and 149 patients were severe leptospirosis. Both pro- and anti-inflammatory cytokines at the time of patient presentation were measured using a multiplex bead array assay. Concentrations of IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-17A, and TNF-α were significantly higher in severe disease compared to mild disease. Among severe patients, levels of IL-6, IL-8 and IL-10, were higher in fatal compared to non-fatal cases. However, only high levels of IL-6 and IL-10, but not TNF-α were independently associated with death after adjustment for age and days of symptoms.This study was one of the biggest studies in the field of cytokines and leptospirosis (33).Chirathaworn also showed that there was no difference of TNF-α level between severe leptospirosis (defined by having any organ dysfunction either by clinical and/or laboratory results as followed; dysfunction was defined as jaundice and/or total bilirubin >3.5 mg/dL or high ALT >120 IU, pulmonary dysfunction was defined as hemoptysis, and/or abnormal chest x-ray or required mechanical ventilation support, AKI was defined as oliguria and/or abnormal creatinine level) and mild leptospirosis patients (34).Mikulski showed the similar finding that there were no significant differences in TNF-α level between the mild and severe group of patients with (35). Moreover, Rizvi et al. reported that TNF-α level was greatly suppressed in most of the leptospira- positive patients with acute liver (36).

Interleukin-6 (IL-6)

IL-6 possesses both pro-inflammatory and anti-inflammatory properties. IL-6 can be secreted by macrophages in response to specific microbial molecules, referred

to as pathogen-associated molecular patterns (PAMPs). These PAMPs bind to an important group of detection molecules of the innate immune system, called pattern recognition receptors (PRRs), including Toll-like receptors (TLRs). These are present on the cell surface and intracellular compartments and induce intracellular signaling cascades that give rise to inflammatory cytokine production. Il-6 can stimulate hypothalamus and inhibits the production of IL-1 and TNF-α (37).High IL-6 concentration was observed in severe leptospirosis and fatal cases (33, 34, 38). Moreover, it is often associated with severe pulmonary haemorrhage syndrome, one of the fatal outcomes in severe leptospirosis (33).In addition, Papa reported that IL-6 levels were elevated at the early stages during severe cases, returning to normal levels at late stages (37).

Therefore, these findings suggest that overproduction of IL-6 could lead to exacerbation of disease and become potentially fatal. In a study by Parsons et al, the authors observed increased levels of interleukin 8 (IL-8) and IL-6 were associated with increased risk of death in patients with acute respiratory distress (39). This might indicate that IL-6 might have direct pathophysiological effect on the severe pulmonary hemorrhage syndrome in severe leptospirosis. Animal data from a mouse model of acute lung injury showed that IL-6 might have direct role in increasing lung inflammation and resulting in poor outcome (40).

Monocyte Chemoattractant Protein -1 (MCP-1)

MCP-1 also known as CCL2, is a key chemokine involved in the migration of monocytes and macrophages to sites of active inflammation. It is a member of the C-C/beta family of cytokines, characterized by the Cys-Cys sequence at its N-terminus. MCP-1 is tethered to endothelial cells via glycosaminoglycans within the plasma membrane. MCP-1 cleavage by MMP-12 is necessary for MCP-1 to interact with its receptor CCR2. MCP-1 activation recruits monocytes to vascular endothelium during active inflammation and also for regular immune surveillance

Papa and Kotrotsiou recruited 42 acute leptospirosis cases. They tested 27 markers of cytokines, chemokines and granulocyte-macrophage colony-stimulating factors (GM-CSF) by using microarray system during an early phase of acute leptospirosis infection. These cytokines include IL-6, IL-8, GM-CSF, vascular endothelial growth factor, MCP-1 and interferon-inducible protein 10 (IP-10).

MCP-1, IP-10 and IL- 6 levels. All of these markers were increased in most of the cases. However, only the severe cases showed significantly higher levels of MCP-1 as compared to the healthy control group (37).

Interleukin 1β (IL-1β)

IL-1β is one of the three member of IL1-gene family which comprised the others two members: IL-1α and IL-1 receptor antagonist (IL-1Ra). Interleukin-1β (IL-1β) is a potent pro-inflammatory cytokine that is crucial for host-defence responses to infection and injury (41). It is also the best characterised and most studied of the 11 IL-1 family members. It is produced and secreted by a variety of cell types although the vast majority of studies have focused on its production within cells of the innate immune system, such as monocytes and macrophages. It is produced as an inactive 31 kDa precursor, termed pro-IL-1β, in response to molecular motifs carried by pathogens called ‘pathogen associated molecular patterns (PAMPs).PAMPs act through pattern recognition receptors (PRR's) on macrophages to regulate pathways that control gene expression (42).

Induction of pro-IL-1β expression is generally referred to as a priming step, and is an inefficient secretion stimulus. The primed cell must now encounter a further PAMP, or DAMP (danger associated molecular pattern, endogenous molecules released from dead cells) to induce the processing and secretion of an active IL-1β molecule.

Limitation of previous studies of cytokines in severe leptospirosis

Possible explanations on the discrepancies among the study of cytokines on severe leptospirosis could be due to many factors. Firstly, the time of cytokines measurement of each study was various and was tested at the different stages of infection. Second, we are still lack of the standard definition of severe leptospirosis. Third, the technique to test cytokines was different such as standard ELISA, or using Luminex assay. Fourth, the gold standard to confirm leptospirosis is different. Some studies used the combination of microscopic agglutination (MAT), culture and polymerase chain reaction (PCR), some studies used only MAT, and some studies used the combination of PCR, MAT, etc. Fifth, most of the studies have the small

number of sample size, and Lastly, most of the studies did not use the multivariate analysis to adjust the confounding factors.

Table 1. Summary studies of cytokines in severe leptospirosis

TNF-α				
Author	Year	No.	Marker	Outcome
Estavoyer et al.	1991	10	TNF-α	The high triglyceride concentrations during episodes of leptospirosis are related to high TNF-a levels. TNF-α was involved with renal impartment and high TNF-α levels were prominent in severe non-fatal leptospirosis (28).
Tajiki and Salomao	1996	6	TNF-α	High level of TNF-α was associated with higher mortality seen in patients suffering from severe leptospirosis (29).
Rizvi et al.	2011	247	IL-8 and TNF-α	IL-8 was found to be elevated (130.81 pg/ml) in a large majority of cases 41/46, 89.1% (P<0.001). TNF-alpha level was largely suppressed (45.63 pg/ml) in most of the leptospira-positive patients in

				comparison with healthy controls (30).
Reis et al.	2013	379	IL-2, IL-4, IL-5, IL10, TNF-α and IFN-c	Severe cases of leptospirosis are differentiated from mild disease by a ‘‘cytokine storm’’ process, and that IL-6 and IL-10 may play an immunopathogenic role in the development of lifethreatening outcomes in human leptospirosis (33).
Rizvi et al.	2014	188	IL-10, IL-8, TNF-α	Ratios of IL-10/TNF-α may play a role in predicting disease prognosis as high IL-10/TNF-α ratios are associated with good prognosis (36).
Mikulski et al.	2015	47	TNF-α, interleukin (IL)-1β, IL-1ra, IL-6, IL-10, interferon-γ (IFN-γ), and the long pentraxin PTX-3	No significant difference was found in the gene expression levels of TNF- α, IL-1β, IL-6, IL-4, IL-12p40, IL-10, IFN-γ. There were no significant differences in TNF-α level between the mild and severe group of patients with leptospirosis (35).

Chirathaworn et al.	2016	40	IL-6, IL-8, IL-10 and TNF-α	TNF-a was induced in leptospirosis patients, there was no statistically difference in patients with and without organ involvement.TNF-a is a cytokine widely studied in immune-mediated diseases including leptospirosis (34).
IL-10 (Interleukin 10)				
Author	Year	No.	Marker	Outcome
Tajiki et al.	1996	12	Ratio of IL-10/TNF- α	The ratio of IL-10/TNF-a correlated with disease severity in that a high ratio was associated with less severe disease and survival (29).
Lowanitchapat et al.	2010	4 Hamster	IL-10, IP-10, TNF-α, TGF-b	TNF-a, TGF-b and IP-10 expression could be demonstrated since day 3 post-infection whereas IL-10 expression was detected later on day 5. Leptospira infection resulted in not only expression of a proinflammatory cytokine, TNF-a, but also a T cell chemokine, IP-10. Detection of IP-10

				suggested.
Kyriakidis et al.	2011	44	TNF-α, sTNFR1, IL-6, IL-8 and IL-10	IL-10/TNF-a ratio was found significantly higher in fatal cases, suggesting an immune system impairment in these cases (30).
Reis et al. 2013	2013	379	IL-2, IL-4, IL-5, IL10, TNF-α and IFN-c	Severe cases of leptospirosis are differentiated from mild disease by a ‘‘cytokine storm’’ process, and that IL-6 and IL-10 may play an immunopathogenic role in the development of lifethreatening outcomes in human leptospirosis (33).
Chirathaworn et al.	2016	40	IL-6, IL-8, IL-10 and TNF-α	Ratio of IL-10/TNF-a in leptospirosis patients with and without organ involvement was not significantly different (p = 0.787).This study supported that IL6, IL-8 and IL-10 are cytokines involved in leptospirosis severity (34).
IL-6 (Interleukin 6)				
Author	Year	No.	Marker	Outcome
Wagenaar et al.	2009	68	ST2 and	Soluble ST2 levels were

			cytokines (TNF-a, IL-1b, IL-6, IL-8, and IL-10)	associated with bleeding and mortality. sST2 levels were significantly correlated with levels of IL-6 (rho 0.45; p = .001), IL-8 (rho 0.72; p,.0001), IL-10 (rho 0.56; p,.0001) also taken at admission High level of IL-6 was often seen in patients with severe leptospirosis and fatal cases (38).
Reis et al.	2013	379	IL-2, IL-4, IL-5, IL10, TNF-α and IFN-c	Among severe patients, levels of IL-6 (P,0.001), IL-8 (P = 0.0049) and IL-10 (P,0.001), were higher in fatal compared to non-fatal cases. High levels of IL-6 and IL-10 were independently associated (P,0.05) with case fatality after adjustment for age and days of symptoms. - IL-6 levels were higher (P = 0.0519) among fatal cases who developed SPHSnthan among who did not (33).
Papa and Kotrotsiou	2015	54 serum samples	27 cytokines in patients with	IL-6 levels were elevated even from day group1

		from 42 patients	acute leptospirosis. (ex. IL-6, IL-8, GM-CSF, IP-10, MCP-1, and VEGF)	(day1-5) in severe cases, and increased further in group 2 (day 6-10), returning to normal levels in group 3 (day11-15). The increased IL-6 levels seen at the early stages, declined in the late phase (37).
MCP-1				
Author	Year	No.	Marker	Outcome
Da Silva et al.	2009	30 mice BALB/c and 30 mice C3H/HeJ	MIP-1 α and MCP-1	MCP-1 levels were observed in both mild and severe patients. Findings from animal study also showed that increased in the MCP-1 expression was well associated with the severity of the disease. The high expression of MCP-1 in all organs of both mice strains at different times of infection. Despite of the increase of MCP-1 in both strains, the effects

				were more preeminent in C3H/HeJ mice, in which the high concentration of MCP-1 was expressed until the sixth day after infection in all organs(43).

TNF-α : tumor necrosis factors-α, MCP-1: monocyte chemoattractant protein-1

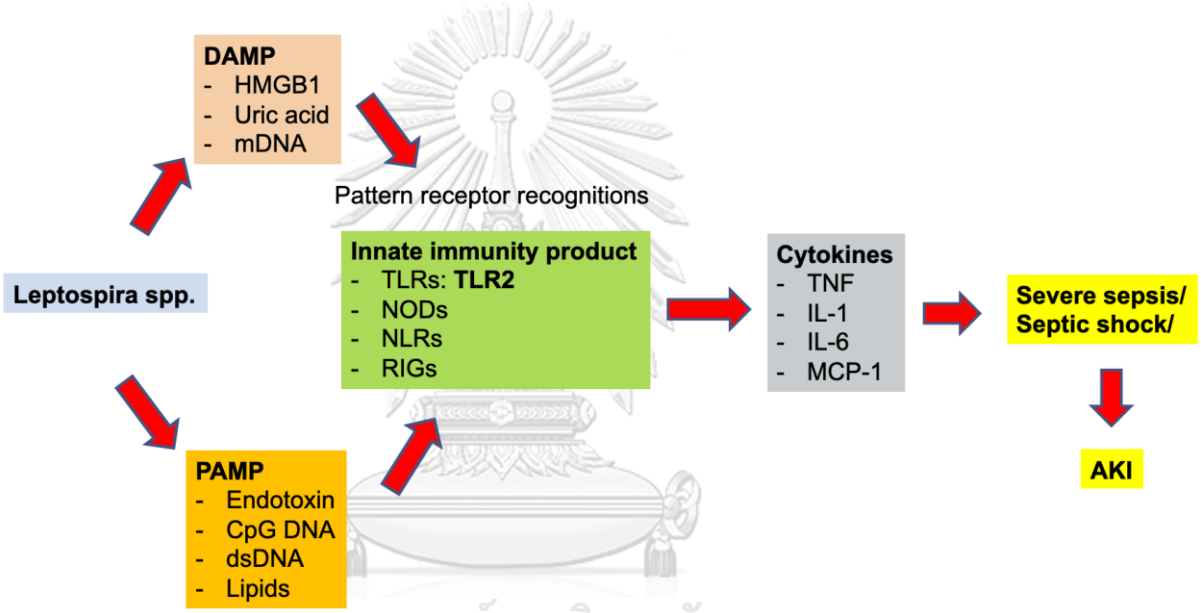


***Figure* 5.** Pathway of "Cytokine Storm" in severe leptospirosis.

Role of immune response

The importance of the adaptive immune response, and in particular the antibody response, in protection from severe leptospirosis is still unknow. While the humoral immune response is accepted widely as the primary mode of immunity to Leptospira infection, a protective role for antibodies has not been demonstrated definitively in humans.

There are only a few studies to explore the role of specific immune response in severe leptospirosis. Lindow S et al enrolled 16 patients hospitalized with acute leptospirosis (13 survivors, 3 fatal cases) and 4 healthy community volunteers for in-depth characterization of clinical course and immune responses. They found that acute phase anti-Leptospira agglutinating antibody titers were lower in non-survivors than

in survivors. This support the animal data in which anti-Leptospira antibodies are critical for bacterial clearance and improved disease outcomes (44, 45).

2.4 Renal manifestation in leptospirosis

Kidney injury is one of the most serious complications of leptospirosis. The incidence of AKI in leptospirosis by using the standard AKI criteria was up to 84% (46). This is approximately two times higher than the AKI incidence in the intensive care unit (ICU) (47). This specific setting injures the kidney by direct effect (direct invasion) and indirect effect such as dehydration, rhabdomyolysis, and bleedin (43). AKI usually develops at the early phase of infection. A recent report by Srisawat et al indicated that most of the patients had AKI on the first day of admission (48). Renal manifestations of leptospirosis associated AKI can vary from mild AKI, to severe AKI, requiring renal replacement therapy (RRT). Not only kidney injury but also tubular dysfunction, mainly at the proximal tubule, is the common feature of leptospirosis associated AKI.

Hypokalemia is one of the most unique features of renal tubular dysfunction laboratory of leptospirosis and is found in nearly 50% of cases. Leptospirosis associated AKI is usually non-oliguric. This finding is independent from its severity, hypercatabolism, rhabdomyolysis, and acidosis (49). Kositseth et al reported that 75% leptospirosis patients had hypermagnesuria, whereas 50% of patients had decreased the threshold of tubular reabsorption of phosphate (50). These abnormal findings were significantly improved within 2 weeks after admission. Alterations such as bicarbonaturia, glycosuria, and reductions in proximal reabsorption of sodium, uric acid, and phosphate excretion have been observed. Moreover, a defect in the urinary concentration can persist for a prolonged period (51). Mild active urinary sediments (pyuria, hematuria, bile pigment, and granular cast) and mild proteinuria (less than 1 gram/day) are usually observed in the urinalysis.

2.5 Management

The early diagnosis and institution of appropriate therapy are the most important points in managing leptospirosis and leptospirosis associated AKI. Sukmark et al proposed the THAI LEPTO score to help the physicians in the rural area to early diagnosis leptospirosis. The score based on the clinical parameters and simple laboratory tests. The simplified score with 7 variables was the summation of

the odds ratio values as follows: hypotension = 3, jaundice = 2, muscle pain = 2, AKI = 1.5, low hemoglobin = 3, hypokalemia with hyponatremia = 3, and neutrophilia = 1. The score showed the highest discriminatory power with area under the curve (AUC) 0.82 (95%CI 0.67±0.97) on fever day 3 and 4 (52). Srisawat et al recently reported the role of urine and plasma neutrophil gelatinase associated lipocalin (NGAL) in early diagnosis of leptospirosis associated AKI. The AUC-ROC values of uNGAL and pNGAL for diagnosis of AKI were 0.91 and 0.92, respectively [49]. Srisawat et al recently reported the role of urine and plasma neutrophil gelatinase associated lipocalin (NGAL) in early diagnosis of leptospirosis associated AKI. The AUC-ROC values of uNGAL and pNGAL for diagnosis of AKI were 0.91 and 0.92, respectively (49).

The use of antibiotics for treating leptospirosis is debatable. A recent meta-analysis has not found sufficient evidence to indicate the use of antibiotics in leptospirosis; however, it was concluded that antibiotic therapy in leptospirosis seemed to have more benefits than drawbacks (53). Antibiotic therapy was associated with a significant reduction in leptospira antigen. Based on the recommendation of the World Health Organization of 2003, severe leptospirosis should be treated with intravenous penicillin (54), ceftriaxone (55), or cefotaxime (56), all equally effective. The duration of antibiotic therapy should be seven days. Oral antibiotics, such as doxycycline, amoxicillin, erythromycin, or azithromycin, are effective in mild cases (57, 58). For prophylaxis of leptospirosis, doxycycline is the mainstay antibiotic.

Recently, Niwatayakul et al has studied the role of corticosteroid and desmopressin in 68 severe leptospirosis patients with pulmonary involvement. Unfortunately, they could not demonstrate the survival benefit of these two treatments compared to control group (59).

2.6 Long term renal outcome after leptospirosis associated AKI

Regarding to KDIGO AKI guideline 2012 recommendations, AKI patients who recover from kidney injury should undergo follow up in the next 3 months. This recommendation is based on an observational study (60). There are a few studies on renal function recovery for tropical infections causing AKI. Daher et al studied 35 patients with leptospirosis associated AKI. The authors tested creatinine clearance, fractional excretion of sodium and potassium, proteinuria, sodium proximal

reabsorption, urinary pH and the ratio of urinary to plasma osmolality (U/Posm) up to 6 months after discharge. Most of tubular function returns to normal except only urinary concentration (43). Herath et al (61) studied leptospirosis associated AKI patients by follow up at least for one year. Only 9% of patients had abnormal renal functions compatible with early stage CKD, but no one needed long term dialysis. Ghasemian conducted a longitudinal prospective study in serologically confirmed leptospirosis associated AKI. Patients with renal failure were followed-up for one year. There was only 2 from 51 cases with persistent serum creatinine higher than 1.5 mg/dL(62). Recently, a community-based study from Taiwan demonstrated the correlation between leptospirosis exposure and CKD. The individuals with previous **l**eptospira exposure had lower glomerular filtration rate and a higher frequency of CKD than those without leptospira exposure. Multivariable analyses confirmed the association between leptospiral infection and lower glomerular filtration rate (63).

Table 2. Summary of follow up studies of leptospirosis associated AKI

Author, year	**N**	**Control**	**AKI Criteria**	**F/U timing**	**Laboratory parameters**	**Findings**
Ghesmian R, 2016	51	No	Cr > 1.5	1 year	Cr	16 had renal dysfunction at discharge, 4 at 3 month and 2 patients at 1 year.
Herath NJ, 2014	44	No	RIFLE criteria	1 year	Cr, GFR	9% had persistent renal dysfunction after 1 year.
Daher EF, 2004	35	Healthy 18 cases	Cr > 1.5	D/C, 3 month, 6 month	sCr, FeNa, FeK, Proteinuria, U/POsm, U pH	U/Posm different between patients and control group at 6 months.

U/P osm: Urine to plasma osmolarity ratio

F/U: follow up

CHAPTER 3: MATERIALS AND METHODS

1. Population and sample

- Target population: Leptospirosis suspicious cases who were admitted into the hospital

- Study population: Leptospirosis suspicious cases who were admitted into the participated hospital

- Sample size

Previous study by Chirathaworn C, Supputtamongkol Y, Lertmaharit S, Poovorawan Y. Cytokine levels as biomarkers for leptospirosis patients. Cytokine. 2016;85:80-2.

$$n = \frac{\sigma^2\left(z_{\frac{\alpha}{2}} + z_{\beta}\right)^2}{e^2}$$

$e = X_1 - X_{2 = 762.5}$

Z_α = Z 0.05/2 = 1.96 (two tail)

mean (SD) IL-6 in severe leptospirosis 849.2 (2042) pg/mL

mean (SD) IL-6 in non-severe leptospirosis 86.7 (116) pg/mL

n = 114

drop out rate at 10%, n = 125

- Study site

Part 1: Multicenter study in Thailand. This study is conducted in 8 provinces around Thailand during August 2012 to November 2014.

1. Prapokklao hospital, Chantaburi
2. Krasang hospital, Buriram,
3. Tungsong hospital, Nakhon Si Thammarat
4. Maharaj Nakhon Si Thammarat hospital, Nakhon Si Thammarat
5. Uttaradit hospital, Uttaradit
6. Nan hospital, Nan
7. Roiet hospital, Roiet

8. Mahasarakarm hospital, Mahasarakarm

Part 2: Multicenter prospective observational study at 15 hospitals in Sisaket province during February 2016 to May 2017.

1.Sisaket hospital

2.Rasi Salai hospital

3.Yang Chum Noi hospital

4.Uthumphon Phisai hospital

5.Huai Thap Than hospital

6.Prong Ku hospital

7.Pha Yu hospital

8.Nam Kliang hospital

9.Si Rattana hospital

10.Non Khun hospital

11.Phu Sing hospital

12.Phrai Bueng hospital

13.Khu Khan hospital

14.Khun Han hospital

15.Kantharalak hospital

2. Inclusion criteria

The patients presenting with clinical suspicious of leptospirosis, high fever (body temperature (BT) higher than 38°c), severe myalgia, and history of exposure to reservoir animals.

3. Exclusion criteria

We excluded the patients who suffered from known other infectious diseases.

4 Assumption

Patients must be older than 18 years old and admitted in participating hospitals. Patients need to meet the inclusion criteria without exclusion criteria.

5. Keywords

Leptospirosis, acute kidney injury, polymerase chain reaction, cytokines

6. Operative definitions

1. Leptospirosis confirmation

We used three standard techniques; microscopic agglutination test (MAT),

direct culture, and PCR technique, to confirm leptospirosis.

We used the term “All cases” for patients who were clinical suspicious of leptospirosis and were enrolled into the cohort. “Leptospirosis cases” were defined if any one of the above tests were positive.

2. Definition of Acute Kidney Injury

We used KDIGO guideline to classify AKI status. Due to the limitation of urine output data, we use only serum creatinine criteria. Definitions of the baseline serum creatinine was the lowest value between the a. the first creatinine recorded on the day of hospital admission or 2) the back calculation from the Modification on Diet in Renal Disease (MDRD) equation for equation for serum creatinine using a GFR of 75mL/min/1.73m^2.

3. Definition of Renal recovery

Recovery was defined as alive and not requiring dialysis during hospitalization and not having a persistent KDIGO stage at hospital discharge (i.e. patients had to improve by at least one KDIGO stage to be considered as recovery)

4. Definition of the first day of enrollment

The first day of enrollment was the first day of clinical suspicious leptospirosis

7. Observation and Measurement

1. Microscopic agglutination test

In brief, MAT was performed by using the standard protocol of the World Health Organization (WHO) guideline (64). A positive MAT was defined as a single serum titer of > 1:400 or a 4-fold rise in pair serum. We used the single serum cut point of > 1:400 based on previous study (65).

2. Culture technique

For direct culture of leptospires, one drop of whole blood was cultured into 4 mL liquid Ellinghausen– McCullough–Johnson– Harris (EMJH) at 29°C for 2 weeks. Detection for leptospires was accomplished by direct observation using Dark-field microscopy (66).

3. Polymerase Chain Reaction

For real time PCR technique, DNA was extracted from blood samples using a High Pure PCR Template Preparation kit (Roche Diagnostics, Germany). The two primers used for amplification of LipL 32 gene were as follows 45F primers (5’ AAG

CAT TAC CGC TTG TGG TG3') and 287R primers (5' CGA ACT CCC ATT TCA GCG AT 3'), PCR reactions of urine samples were performed in a final volume of 20 µ l, corresponding to 2 µ l of genomic DNA and 18 µ l of reaction mix containing 25 mM of each dNTP; 0.1 µ l of Taq DNA polymerase; 0.4 µ l of each primer in 25 mM MgCl2 and 10x KCl under 13.5 µ l DW. The PCR program consisted of an initial cycle of 94°C for 10 min, followed by 40 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min and a final extension step at 72°C for 7 min. PCR products were run on a 1% agarose gel with ethidium bromide and photographed.

4. Real time Polymerase Chain Reaction

- Calibration Curve

The concentration of genomic DNA (gDNA) extracted from Leptospira interrogans was measured by a spectrophotometer (ND-1000 Nanodrop, Nanodrop Technologies, and Wilmington, DE, USA). Calibration Curve was created from a 1X104 copies/mL followed by a 10-fold serial dilution to 1X100 copies/mL (67).

- Quantitative Real Time PCR Method

Collecting whole blood from EDTA 200 microlitre (µL) of clinical sample. DNA was extracted by using a High Pure PCR Template Preparation kit (Roche Diagnostics, Germany), eluting with 50 uL elution buffer. The primers used for amplification of LipL32 gene were as follows LipL32-45F (5'-AAG CAT TAC CGC TTG TGG TG-3') and LipL32-286R (5'-GAA CTC CCA TTT CAG CGA TT-3'). The fluorescent probe was LipL32-189P (FAM-5'-AA AGC CAG GAC AAG CGC CG-3'-TAMRA). A final volume of 20 µL contained 5 µL of genomic DNA and 15 µL of reaction mix (10 µL of 2X TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 1 µL of each 10 µM primer, 0.4 µL of 10 µM probe under 2.6 µL Distilled Water). The PCR program consisted of 45 cycles, each cycle consisting of 95°C for 15 seconds and 60°C for one minute. Positive and negative controls were included in every experiment. Results were read by threshold cycle (Ct) value (68).

5. Multiplex bead assay

Cytokine Level (IL-1β, IL-6, TNF-α, MCP-1) in serum measurement by ELISA kit (Magnetic Luminex® Performance Assay, R&D System, MN)

5. Urine and plasma NGAL measurement

Urine NGAL was measured by ELISA (R&D, Minneapolis, MN, USA), following the manufacturers' instructions. Plasma NGAL was tested using the Triage NGAL kit (Alere, San Diego, CA, USA). Both biomarkers were tested on the first day of enrollment. All samples were analyzed in duplicate.

8. Research design

Thai Lepto AKI study was a multicenter, prospective, cohort study of patients presenting with clinical suspicion of leptospirosis. The study composed of two main cohorts.

The first cohort was conducted in 9 centers in 8 provinces around Thailand during August 2012 to November 2014. Of the nine participating hospitals, 2 centers are from the Northern part, 3 centers from the Northeastern part, 1 center from the Eastern part and the other three centers from the Southern part. For the level of care, 2 centers were the referral/tertiary care hospital, 7 centers were the provincial hospital.

The second cohort was studied at 15 hospitals in Sisaket provinces, Thailand during February 2016 to July 2017. All cases were hospitalized. The patients need to be primary admitted into the participating centers and were not diagnosed with other infectious diseases at any time. For the level of care, 1 center was the referral/tertiary care hospital, 14 centers were the provincial hospital.

Sample collection

First cohort: A 12-ml blood sample was taken and well mixed on the day 1, day 2, day 3 and day 7 after enrollment. The first day of enrollment was the first day of clinical suspicious leptospirosis. A 30-ml urine sample was obtained on the same day. Urine samples were poured into 50-ml conical centrifuge tubes. Both of plasma and urine were centrifuged for 10 minutes at 1000 G at 4°C, and frozen at -20°C until shipped to the central laboratory. Samples were then stored at -80°C until analyzed.

Second cohort: A 12-ml blood sample was taken and well mixed on the day 1and day 7 after enrollment. Both of plasma and urine were centrifuged for 10 minutes at 3000. Samples were then stored at -80°C until analyzed.

9. Ethical considerations

Investigators concern about the rights of patients. Before recruitment, investigators have given all patients or caregivers both oral and written research information until patients or caregivers fully understand and can make a decision to

be or not to be participated in the study according to their willingness. Patients can leave the study anytime without effect on the regular therapy. All data will be kept confidential and presented by concealing patients' private profile. Study protocol is approved by the Institutional Review Board of Faculty of Medicine, Chulalongkorn University, and the Institutional Review Board of Ministry of Public Health of Thailand.

10. Expected benefits and applications

This study will contribution the information about the role of cytokines in pathogenesis of leptospirosis associated AKI. Currently, we have effective intervention to modify the cytokine response and never been tested in this specific population. This data may be used to define the target intervention to prevent or improve outcome of leptospirosis associated AKI in the future.

11. Obstacles, strategies to solve the problems

There are several possible limitations to our study. First, due to the study design and variable timing of hospital admission, we cannot test all patients from the first day of fever. Therefore, most of our patients had increased serum creatinine since the first day of enrollment. Therefore, the interpretation of the result for the role of cytokines and pathogenesis of leptospirosis AKI need to understand this limitation. However, will stratify patients by day of fever to reduce this lead time bias. Second, we are unable to measure process of care variables such as the effect of co-interventions on cytokines level and its influence on development of AKI.

12. Administration and Time schedule

	2015	2016									
	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct
1. Review literature	x	x									
2. Patients recruitment and data collection			x	x	x						
3. Measurement cytokines panel						x	x	x			
4. Data analysis									x	x	
5. Manuscript preparation											x

13. Budgets

Detailed budget

Items	**Budget (Bath)**
1. Travelling expense for collecting data (including aircraft fare, accommodation)	50000
2. Testing samples, Salary for nurse coordinators, nurses who register data, statistician	400000
3. Cost for operational the project computer, internet access, office equipment	30000
4. Miscellaneous	20000
Total	500000

14. Statistical analysis

Clinical characteristics and biomarkers on the day of enrollment were compared between patients with AKI and those without AKI and between renal recovery patients and non-recovery patients at hospital discharge time. Categorical data were expressed as proportions and compared using a Chi-square test. Continuous data were expressed as mean ± standard deviation and compared using the student' s t-test or were expressed as median ± intra-quartile Biomarkers and Leptospirosis AKI range (IQR) and compared using Mann-Whitney U test, as appropriate. We fitted an inclusive logistic regression model using biomarkers to predict the probability of AKI and recovery from AKI, and expressed these results as odds ratios (OR), and 95% confidence intervals (CI). To assess the predictive ability of single and multiple biomarkers, receiver operating characteristic (ROC) curves were generated and the area under the curve (AUC) was calculated. Statistical analyses were performed using SPSS version 17.0 and GraphPad PRISM version 5.1. P values < 0.05 were considered to indicate statistical significance.

CHAPTER 4: RESULTS

4.1 Patients clinical characteristics

In the first cohort, of the 221 subjects with clinical suspicion of leptospirosis (all cases), urine samples were unavailable in 10. These cases and 5 additional patients that could not be identified for AKI status were excluded (Figure 6). Of the remaining 206, 55 (26%) were diagnosed with AKI. Of the 55 cases of AKI, 7.3% had KDIGO stage1, 16.4% had KDIGO stage2, and 76.4% had KDIGO stage3. AKI patients had the recovery rate at 81.8% (Figure 6). Twenty nine percent of AKI patients required dialysis support. AKI patients had mortality rate 10.1% while non-AKI patients had only 0.6%. AKI patients had lower body temperature, more leukocytosis, lower hemoglobin, more thrombocytopenia, higher bilirubin (TB/DB), higher SGOT/SGPT, and lower sodium than non-AKI patients (Table 3). The diagnosis of leptospirosis was confirmed in 113 patients (54.9%), and about one third (37.2%) developed AKI. Of the 42 AKI cases, most of them had KDIGO stage 3 (33 cases, 78.6%), followed by KDIGO stage 2 (7 cases, 16.7%), and KDIGO stage 1 (2 cases. 4.7%). Twenty four percent of AKI patients need dialysis support. The mortality rate in AKI patients was 9.5% and no one in non-AKI patients was died. Again, Leptospirosis patients who developed AKI were likely to have more leukocytosis, more thrombocytopenia, lower hemoglobin, more thrombocytopenia, higher bilirubin level, and higher serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) than non-AKI patients (Table 3). By contrast, only 13 from the 93 (14.0%) non-leptospirosis patients, developed AKI (Figure 6). Of the 13 AKI cases, 15.4% had KDIGO stage1 and KDIGO stage 2. While 69.2% had KDIGO stage 3. Forty six percent of AKI patients required dialysis support. AKI patient had higher mortality rate than non-AKI patients, 15.4% vs 1.2%. Patients with AKI had lower diastolic blood pressure, more thrombocytopenia, higher bilirubin (TB/DB), and higher SGOT than non-AKI patients (Table 3).

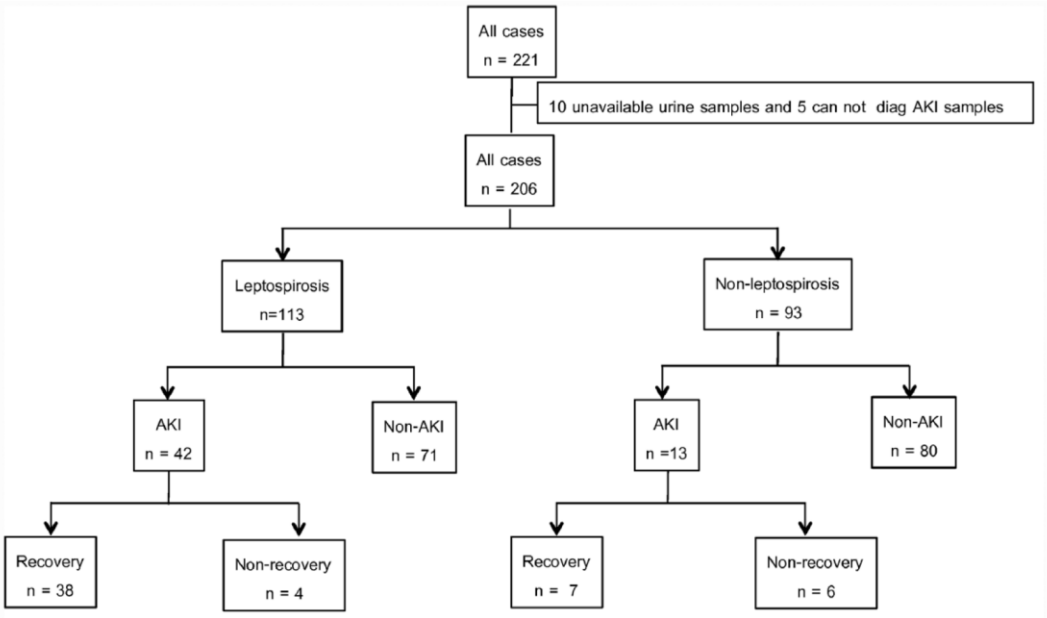


Figure 6. Subject disposition for the first Thai lepto AKI study cohort

Characteristic	All cases (n=206)			Leptospirosis cases (n=113)			Non-leptospirosis cases (n=93)		
	AKI (n=55)	Non-AKI (n=151)	P-value	AKI (n=42)	Non-AKI (n=71)	p-value	AKI (n=13)	Non-AKI (n=80)	p-value
Gender, Male (%)	44(81.5)	119(78.3)	0.70	35(85.4)	59(81.9)	0.80	9(69.2)	60(75.0)	0.74
Fever day, days	4.5(2.1)	3.9(2.4)	0.08	4.7(2.2)	3.8(2.6)	0.21	3.6(1.6)	3.94(2.3)	0.65
Age, years	46.3(17.1)	41.6(14.8)	0.06	43.0(12.9)	36.6(11.1)	0.17	46.1(22.6)	35.3(16.2)	0.28
Body temperature, °c	37.1(2.2)	38.4(1.1)	0.001	37.4(1.1)	38.0(1.1)	0.07	36.3(4.0)	38.78(1.1)	0.003
SBP, mmHg	107.8(21.2)	111.7(20.0)	0.30	109.1(22.9)	110.2(18.6)	0.89	104.1(16.2)	114.9(21.9)	0.06
DBP, mmHg	64.6(15.0)	68.9(12.9)	0.06	65.4(15.2)	68.8(13.2)	0.35	62.3(14.9)	69.5(13.1)	0.039
Creatinine, mg/dl	5.0(1.9)	1.0(0.3)	0.009	5.1(2.2)	1.0(0.6)	<0.001	4.1(3.5)	0.9(0.4)	<0.001
WBC x 103,	12.7(10.6)	7.9(4.9)	<0.001	13.3(9.8)	7.9(8.1)	0.002	9.3(10.6)	7.7(5.1)	0.10
Hgb, g/dl	11.3(2.9)	12.4(3.4)	<0.001	11.0(1.9)	11.6(2.8)	0.019	13.2(3.2)	12.2(2.0)	0.56
Platelet x 103	88.6(85.5)	166.8(114.0)	0.001	39.0(91.0)	150.5(90.3)	<0.001	68.5(109.0)	139.0(117.3)	0.002
TB, mg/dl	4.0(6.9)	1.2(2.9)	<0.001	4.3(13.1)	1.2(3.7)	0.007	3.9(4.4)	1.3(1.5)	0.04
DB, mg/dl	2.3(4.9)	0.3(1.3)	<0.001	2.3(5.5)	0.3(1.4)	<0.001	2.0(3.1)	0.5(0.7)	0.007
SGOT, u/L	61(124)	49(71)	<0.001	58(75)	49(81)	0.037	544(2770)	47(85)	0.03
SGPT, u/L	64(87)	46(70)	0.009	61(80)	46(80)	0.05	209(5393)	44(83)	0.09
Na, mEq/L	133.0(8)	135.0(7)	0.023	134.8(4.4)	134.5(4.1)	0.61	134.5(5.8)	128.6(28.7)	0.51
K, mEq/L	4.0(1.1)	3.5(0.5)	0.56	3.8(0.5)	3.6(0.6)	0.28	4.6(1.9)	3.36(0.5)	0.09
HCO3, mEq/L	19.0(4.4)	22.1(7.0)	0.06	19.8(4.3)	22.0(7.5)	0.28	16.5(4.0)	21.9(6.6)	0.001

Table3. P Patients characteristics by AKI status on the first day of enrollment into the study

SBP: systolic blood pressure, DBP: diastolic blood pressure, WBC: white blood cell, Hgb: hemoglobin, TB: total bilirubin, DB: direct bilirubin, SGOT: Serum Glutamic Oxaloacetic Transaminase, SGPT: Serum Glutamic, Pyruvic Transaminase, HCO3; bicarbonate Values in the table is mean (s.d.) for Fever day, Age, Body temperature, SBP, DBP, Hgb, Na, K, HCO3; median (IQR) for Creatinine, WBC, Platelet, TB,DB, SGOT, SGPT and count (%) for Gender, and RRT, RRT: renal replacement therapy.

4.2 Cytokines concentrations by AKI status

We used the first cohort to test the role of biomarkers and cytokines concentration in leptospirosis associated AKI. Of 206 cases, cytokines concentrations were tested in 191 cases, 108 cases were leptospirosis confirmed cases, and 83 cases were non-leptospirosis cases. Firs focusing on all cases with suspected leptospirosis, AKI patients had significantly higher plasma IL-6, MCP-1, TNF-α, and IL-1β than non-AKI patients on the 1st day of enrollment, IL-6; AKI vs non-AKI 13.0 ng/mL (IQR 60.6) vs 2.8 pg/mL (IQR 24.0), P = 0.019, MCP-1: AKI vs non-AKI 314 pg/mL (IQR 820.9) vs 101.7 pg/mL (IQR 223.8), P < 0.001, TNF-α: AKI vs non-AKI 523.3 pg/ml (IQR 2217.2) vs 136.9 pg/mL (IQR 542.0), P < 0.001, IL-1β: AKI vs non-AKI 45.4 pg/ml (IQR 317.5) vs 16.2 pg/mL (IQR 24.9), P < 0.001 (Table 4).

Cytokine	AKI			Non-AKI			p-value between AKI vs Non-AKI [b]		p-value of change from day 1 to day 7 between AKI vs Non-AKI [b]
	Day 1 (N=53)	Day 7 (N=33)	p-value within group [a]	Day 1 (N=139)	Day 7 (N=74)	p-value within group [a]	Day 1	Day 7	
IL-6, pg/mL, (Q1, Q3)	13.0 (2.8, 63.4)	2.8 (2.8, 2.8), N=22	<0.001	2.8 (2.8, 26.8)	2.8 (2.8, 2.8), N=54	0.714	0.019	0.357	0.008
MCP-1, pg/mL, (Q1, Q3)	314.6 (116.3, 937.2)	9.1 (4.1, 34.6), N=32	<0.001	101.7 (20, 243.8)	5.6 (2, 5.6), N=62	<0.001	<0.001	0.003	<0.001
TNF-α, pg/mL, (Q1, Q3)	523.3 (74.5, 2291.7)	104.1 (88.8, 171.8)	<0.001	136.9 (25.1, 567.1)	106.4 (79.4, 144)	<0.001	<0.001	0.434	0.001
IL-1β, pg/mL, (Q1, Q3)	45.4 (19, 336.5)	7.9 (5.5, 11.7)	<0.001	16.2 (7, 31.9)	5.4 (5.4, 6.8)	<0.001	<0.001	<0.001	0.001

[a] Comparing within group using Wilcoxon signed-rank test

Table 4. Cytokine level on the first day of enrollment day 7 between AKI and non-AKI in all patients (n=192)

For patients with confirmed leptospirosis, median MCP-1, and TNF-α on the 1st day of enrollment were also significantly higher for patients with AKI: MCP-1, AKI vs non-AKI 309.3 pg/mL (IQR 816.2) vs 138.8 ng/ml (IQR 220.7), P = 0.003; TNF-α, AKI vs non-AKI 492.3 (IQR 2145.9) vs 77.5 (IQR 729.3), P = 0.003. While there was no significant difference of IL-6 and IL-1β between AKI and non-AKI patients (Table 5).

Cytokine	AKI			Non-AKI			p-value between AKI vs Non-AKI [b]		p-value of change from day 1 to day 7 between AKI vs Non-AKI [b]
	Day 1 (N=53)	Day 7 (N=33)	p-value within group [a]	Day 1 (N=139)	Day 7 (N=74)	p-value within group [a]	Day 1	Day 7	
IL-6, pg/mL, (Q1, Q3)	23.4 (2.8, 63.4)	2.8 (2.8, 2.8), N=16	0.003	9.4 (2.8, 47.2)	2.8 (2.8, 2.8), N=22	0.004	0.489	0.678	0.179
MCP-1, pg/mL, (Q1, Q3)	309.3 (121, 937.2)	7.2 (2.9, 22.4), N=24	0.001	138.8 (58.3, 279)	5.6 (1.6, 5.6), N=26	<0.001	0.003	0.041	0.134
TNF-α, pg/mL, (Q1, Q3)	492.3 (68.6, 2204.5)	104.1 (88.8, 171.4)	<0.001	77.5 (20.7, 749.3)	105.6 (63.8, 138.5)	<0.001	0.003	0.443	0.244
IL-1β, pg/mL, (Q1, Q3)	40.5 (19, 336.5)	7.6 (5.4, 10.7)	<0.001	20.7 (14.4, 97.7)	5.4 (5.4, 6.3)	<0.001	0.090	0.001	0.344

[a] Comparing within group using Wilcoxon signed-rank test

Table 5. Cytokines on the first day of enrollment and day7 between AKI and non-AKI in leptospirosis confirmed patients (n=111)

For patients without leptospirosis, median MCP-1, TNF-α, and IL-1β on the 1st day of enrollment were also significantly higher for patients with AKI: MCP-1, AKI vs non-AKI 436.8 pg/mL (IQR 2176.9) vs 28.1 ng/ml (IQR 162.4), P < 0.001; TNF-α, AKI vs non-AKI 1129.8 (IQR 5788.5) vs 150.5 (IQR 420.1), P = 0.008; IL-1β, AKI vs non-AKI 62.0 (IQR 510.5) vs 9.5 (IQR 13.8), P < 0.001. While there was no significant difference of IL-6 between AKI and non-AKI patients (Table 6). Figure 8, 9 and 10 showed level of IL-6, MCP-1, TNF-α, and IL-1β stratified by AKI status, respectively.

Cytokine	AKI			Non-AKI			p-value between AKI vs Non-AKI [b]		p-value of change from day 1 to day 7 between AKI vs Non-AKI [b]
	Day 1 (N=53)	Day 7 (N=33)	p-value within group [a]	Day 1 (N=139)	Day 7 (N=74)	p-value within group [a]	Day 1	Day 7	
IL-6, pg/mL, (Q1, Q3)	5.6 (2.8, 92.3)	2.8 (2.8, 2.8), N=6	0.026	2.8 (2.8, 7.6)	2.8 (2.8, 2.8), N=33	0.635	0.147	0.265	0.044
MCP-1, pg/mL, (Q1, Q3)	436.8 (96.6, 2273.5)	32.2 (5.9, 43.7)	0.012	28.1 (9.3, 171.7)	5.6 (3.3, 5.6), N=37	<0.001	<0.001	0.005	0.008
TNF-α, pg/mL, (Q1, Q3)	1129.8 (225, 5813.5)	127 (86.8, 200.2)	0.012	150.5 (75.2, 495.3)	106.7 (81.6, 166.2)	<0.001	0.008	0.604	0.062
IL-1β, pg/mL, (Q1, Q3)	62 (23.5, 534)	10.1 (7.5, 12)	0.012	9.5 (5.7, 19.5)	5.4 (5.4, 7)	<0.001	<0.001	0.005	0.009

[a] Comparing within group using Wilcoxon signed-rank test

Table 6 Cytokines level on the first day of enrollment and day 7 between AKI and non AKI in non-leptospirosis patients. (n=81)

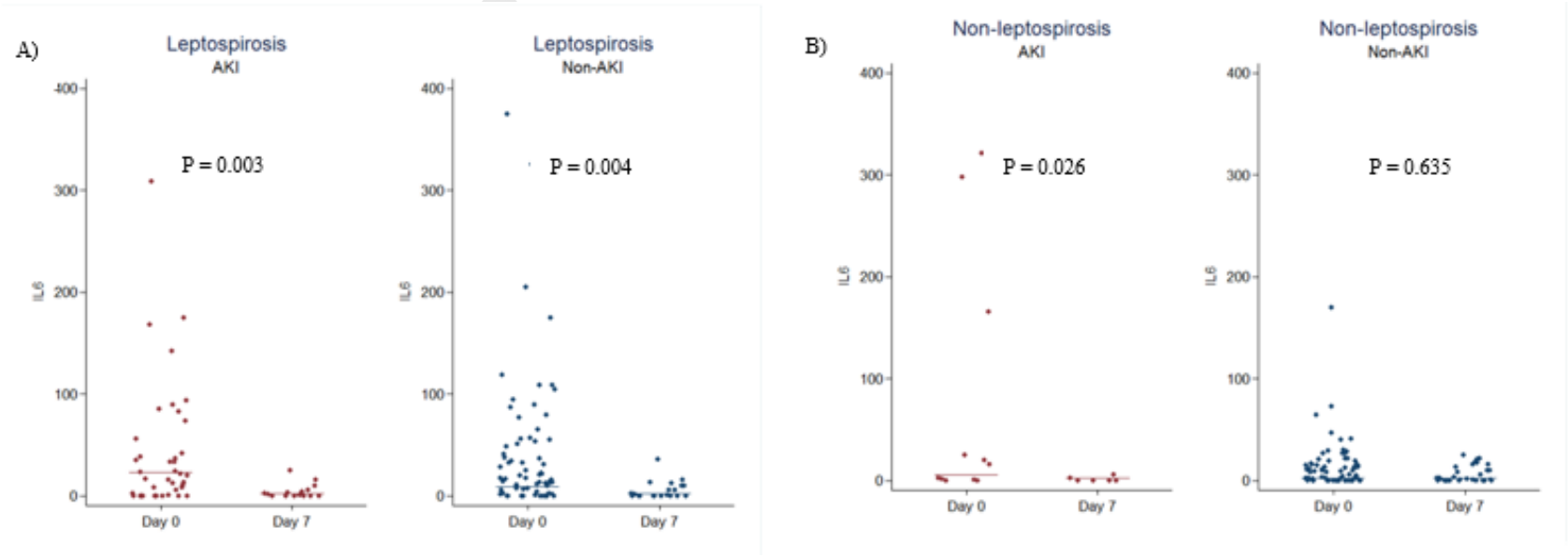


Figure 7. IL-6 level by AKI status in A) leptospirosis confirmed cases and in B) non-leptospirosis cases

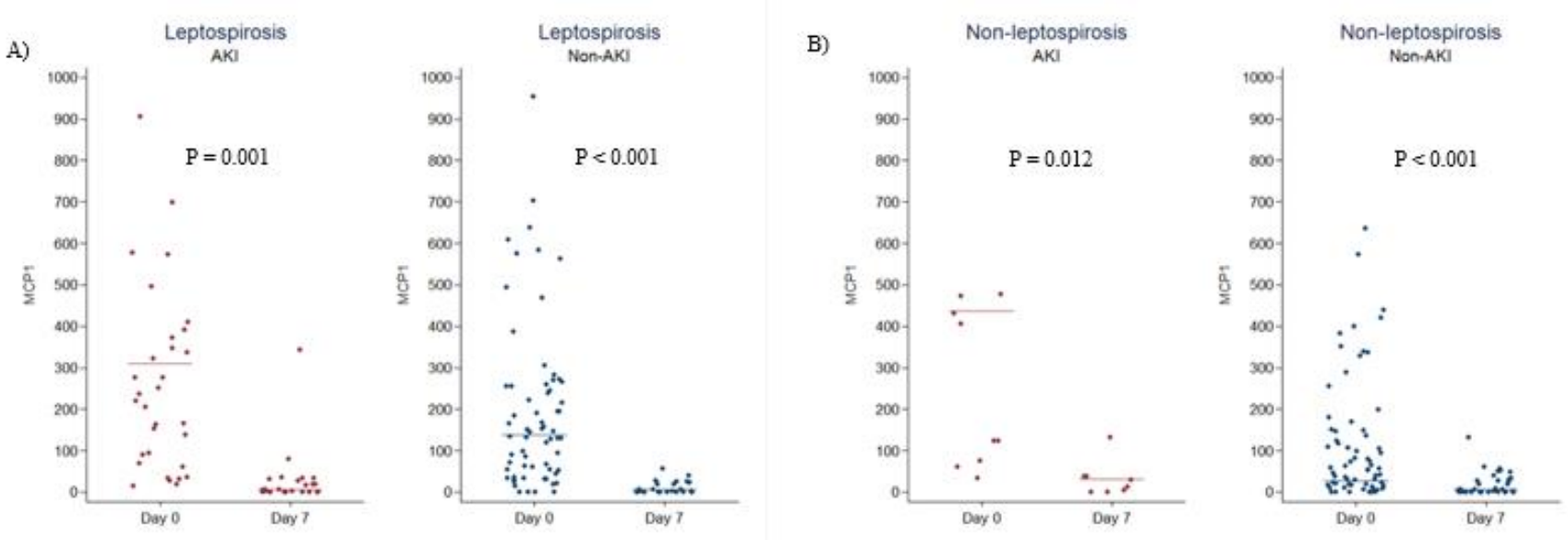


Figure 8. MCP-1 level by AKI status in A) leptospirosis confirmed cases and in B) non-leptospirosis cases

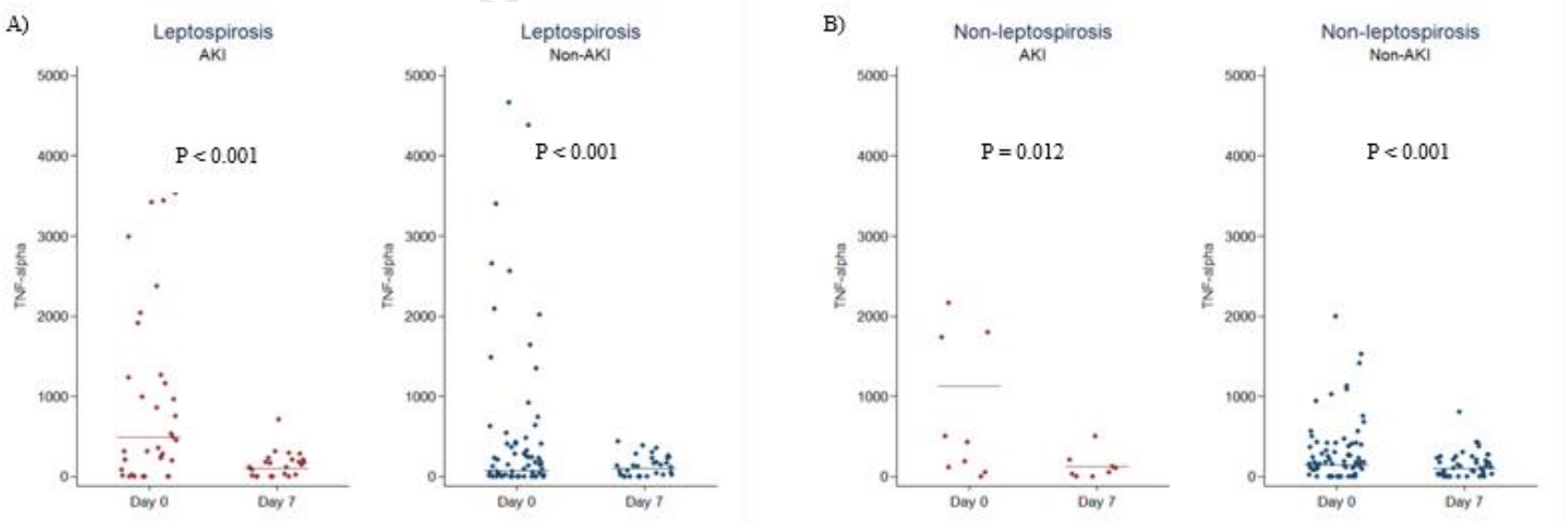


Figure 9. TNF-α level by AKI status in A) leptospirosis confirmed cases and in B) non-leptospirosis cases

4.3 Cytokines concentrations by 1renal recovery status

In the recovery group, there was significant decreased of all cytokines (all patients group), only MCP-1, TNF-α, and IL-1β (both leptospirosis confirmed cases and non-leptospirosis cases) from the first day of enrollment and day 7, while there was no change of all cytokines level in the non-recovery group (all patients, leptospirosis confirmed cases, and non-leptospirosis cases). However, when compare the level of cytokines between recovery group and non-recovery group, there was no difference of cytokines between group on the first day and on day 7 after enrollment in all cases, leptospirosis confirmed cases, and non-leptospirosis cases (Table 7-9).

Cytokine	Recovery			Non-recovery			p-value between Recovery vs Non-recovery [b]		p-value of change from day 0 to day 7 between Recovery vs Non-recovery [b]
	Day 1 (N=44)	Day 7 (N=29)	p-value within group [a]	Day 1 (N=9)	Day 7 (N=4)	p-value within group [a]	Day 0	Day 7	
IL-6, pg/mL, (Q1, Q3)	15.4 (2.8 – 65.3)	2.8 (2.8 – 2.8), N=19	0.017	12.7 (2.8 – 41.4)	2.8 (2.8 – 2.8), N=3	0.166	0.864	0.670	0.574
MCP-1, pg/mL, (Q1, Q3)	327.6 (118.7 – 1098.2)	7.6 (4.1 – 32.2), N=28	<0.001	207.3 (96.7 – 468.7)	21.3 (6.1 – 38.8)	0.068	0.687	0.493	0.458
TNF-α, pg/mL, (Q1, Q3)	631.6 (79.2 – 2248.1)	104.1 (88.8 – 171.4)	<0.001	523.3 (21.0 – 8775.2)	146.6 (93.8 – 233.5)	0.068	0.758	0.408	0.741
IL-1β, pg/mL, (Q1, Q3)	44.9 (19.0 – 296.6)	7.9 (5.5 – 11.2)	<0.001	45.4 (9.6 – 607.0)	9.1 (6.0 – 12.0)	0.068	0.722	0.934	0.699

[a] Comparing within group using Wilcoxon signed-rank test

Table 7. Cytokines level on the first day of enrollment and day 7 between recovery and non-recovery in all AKI patients (n=53)

Cytokine	AKI			Non-AKI			p-value between AKI vs Non-AKI [b]		p-value of change from day 0 to day 7 between AKI vs Non-AKI [b]
	Day 1 (N=37)	Day 7 (N=23)	p-value within group [a]	Day 1 (N=4)	Day 7 (N=2)	p-value within group [a]	Day 1	Day 7	
IL-6, pg/mL, (Q1, Q3)	15.4 (2.8 – 65.3)	2.8 (2.8 -2.8), N=15	0.054	18.1 (7.8 – 32.4)	2.8 (2.8 -2.8), N=1	0.317	0.685	0.776	NA
MCP-1, pg/mL, (Q1, Q3)	327.6 (118.7 – 1098.2)	5.6 (2.3 – 19.9), N=22	<0.001	202.5 (147.2 – 338.0)	21.3 (10.8 – 31.8)	0.180	0.429	0.250	NA
TNF-α, pg/mL, (Q1, Q3)	631.6 (79.2 – 2248.1)	104.1 (80.2 – 171.4)	<0.001	572.8 (19.5 – 4949.9)	190.8 (103.9 – 277.7)	0.180	0.455	0.455	NA
IL-1β, pg/mL, (Q1, Q3)	44.9 (19.0 – 296.6)	7.9 (5.4 – 11.9)	<0.001	25.8 (5.8 – 326.2)	6.0 (5.4 – 6.5)	0.180	0.455	0.290	NA

[a] Comparing within group using Wilcoxon signed-rank test

Table 8. Cytokines level on the first day of enrollment and day 7 between recovery and non-recovery in all AKI patients (n=41)

4.4 Cytokines and AKI biomarkers level by day of fever

We have also stratified the level of biomarkers by day of fever (first 3 days, and after day 3 of fever) to decrease the lead time bias. For all cases, we significantly found uNGAL, pNGAL, MCP-1, TNF-α, and IL-1β level of AKI group higher than non-AKI group on the first three day of fever. From day 4 after onset of fever, all biomarkers and cytokines still showed the higher level in AKI group than in non-AKI group.

For the leptospirosis confirmed patients, we also found found uNGAL and pNGAL level of AKI group higher than non-AKI group on the first three day of fever and after day 3 of fever. MCP-1 level after day 3 of fever and TNF-α on the first three day of fever showed the higher level in AKI group than in non-AKI group.

For the non-leptospirosis patients, we also found found uNGAL and pNGAL level of AKI group higher than non-AKI group on the first three day of fever and after day 3 of fever. Only MCP-1 level on the first three day of fever and IL-1β both on the first three day of fever and after three days of fever showed the higher level in AKI group than in non-AKI group. (Table 8)

Cytokine	AKI			Non-AKI			p-value between AKI vs Non-AKI [b]		p-value of change from day 0 to day 7 between AKI vs Non-AKI [b]
	Day 1 (N=7)	Day 7 (N=6)	p-value within group [a]	Day 0 (N=5)	Day 7 (N=2)	p-value within group [a]	Day 1	Day 7	
IL-6, pg/mL, (Q1, Q3)	2.8 (2.8 – 26.3)	2.8 (2.8 – 2.8), N=4	0.162	8.3 (2.8 – 315.1)	2.8 (2.8 – 2.8)	0.317	0.435	NA	NA
MCP-1, pg/mL, (Q1, Q3)	431.3 (116.3 – 4040.0)	32.2 (6.3 – 41.5)	0.028	442.2 (76.9 – 492.4)	23.6 (1.4 – 45.9)	0.180	0.935	0.739	NA
TNF-α, pg/mL, (Q1, Q3)	1736.3 (157.5 – 2291.7)	127.0 (89.8 – 211.0)	0.028	523.3 (292.6 – 9335.2)	136.6 (83.8 – 189.4)	0.180	0.935	0.935	NA
IL-1β, pg/mL, (Q1, Q3)	54.4 (29.4 – 119.9)	8.5 (7.1 – 11.2)	0.028	69.6 (17.7 – 948.2)	12.0 (11.7 – 12.4)	0.180	0.808	0.182	NA

[a] Comparing within group using Wilcoxon signed-rank test

Table 9. Cytokines level on the first day of enrollment and day 7 between recovery and non-recovery in all AKI patients (n=12)

Table 10. Analysis of biomarkers stratified by day of fever between AKI and non-AKI (A) in all cases, (B) in leptospirosis cases, and (C) in non-leptospirosis cases.

Biomarkers	Day of fever	AKI	Non-AKI	P-value
A) Concentration of biomarkers between AKI and non-AKI stratified by day of fever in all cases (N=206 but N=191 for the last 4 cytokines)				
uNGAL, ng/ml	day 1 – day 3	179.4 (4733.9)	16.1 (35.9)	<0.001
	day 4 up	562.0 (688.4)	21 (48.0)	<0.001
pNGAL, ng/ml	day 1 – day 3	891.7 (682.0)	102 (131.5)	<0.001
	day 4 up	1300.0 (644.0)	173 (221.5)	<0.001
Serum creatinine, mg/dl	day 1 – day 3	3.60 (2.95)	0.96 (0.32)	<0.001
	day 4 up	4.54 (2.86)	0.88 (0.38)	<0.001
IL6, pg/mL	day 1 – day 3	5.8 (2.8 – 34.0)	3.7 (2.8 – 28.5)	0.875
	day 4 up	23.4 (2.8 – 95.6)	2.8 (2.8 – 15.5)	0.045
MCP1, pg/mL	day 1 – day 3	372.9 (131.5 – 1215.6)	133.9 (23.4 – 261.5)	0.002
	day 4 up	300.8 (76.9 – 497.0)	61.1 (21.3 – 216.2)	0.001
TNF-α, pg/mL	day 1 – day 3	1153.0 (320.9, 2053.8)	118.8 (22.7, 495.3)	0.001
	day 4 up	322.3 (48.2, 3613.5)	153.1 (25.1, 749.3)	0.039
IL1-β, pg/mL	day 1 – day 3	51.0 (28.4, 203.5)	17.8 (6.9, 28.1)	0.003
	day 4 up	30.4 (13.4, 607)	15.1 (7.7, 36.6)	0.033
B) Concentration of biomarkers between AKI and non-AKI stratified by day of fever in leptospirosis cases (N=113 but N=108 for the last 4 cytokines)				
uNGAL, ng/ml	day 1 – day 3	158.4 (867.7)	20.8 (45.7)	<0.001
	day 4 up	550.3 (583.3)	23.2 (64.4)	<0.001
pNGAL, ng/ml	day 1 – day 3	945 (732)	111 (134)	0.001
	day 4 up	1300 (593.5)	211.5 (147.3)	<0.001
Serum creatinine, mg/dl	day 1 – day 3	3.6 (2.95)	0.98 (0.32)	<0.001
	day 4 up	5.1 (2.5)	0.88 (0.45)	<0.001
IL6, pg/mL	day 1 – day 3	2.8 (2.8, 37.5)	28 (2.8, 49.1)	0.187
	day 4 up	31.9 (2.8, 71.5)	6.1 (2.8, 35)	0.277
MCP1, pg/mL	day 1 – day 3	314.6 (96.7, 937.2)	166.4 (78.7, 286.6)	0.079
	day 4 up	275.4 (121, 468.7)	122.4 (45.5, 216.2)	0.035
TNF-α, pg/mL	day 1 – day 3	924.1 (230.6, 2204.5)	46.7 (20.7, 428.6)	0.006
	day 4 up	322.3 (48.2, 3168.7)	153.9 (25.1, 1387.7)	0.273
IL1-β, pg/mL	day 1 – day 3	42.1 (19, 336.5)	20.7 (17.7, 36.8)	0.099
	day 4 up	24.7 (13.4, 480.3)	21.3 (13.2, 213.6)	0.628
C) Concentration of biomarkers between AKI and non-AKI stratified by day of fever in non-leptospirosis cases (N = 93 but N=83 for the last 4 cytokines))				
uNGAL, ng/ml	day 1 – day 3	4793.2 (7941.5)	13.3 (20.6)	0.001
	day 4 up	1403.1 (2355.6)	16.8 (17.7)	<0.001
pNGAL, ng/ml	day 1 – day 3	725 (818.8)	97.5 (134.25)	0.001

	day 4 up	391 (1112)	100 (133.25)	0.002
Serum creatinine, mg/dl	day 1 – day 3	2.36 (3.68)	0.91 (0.36)	<0.001
	day 4 up	4.22 (5.28)	0.88 (0.28)	<0.001
IL6, pg/mL	day 1 – day 3	8.9 (2.8, 26.3)	2.8 (2.8, 5.9)	0.167
	day 4 up	5.6 (2.8, 315.1)	2.8 (2.8, 8.5)	0.314
MCP1, pg/mL	day 1 – day 3	431.3 (146.7, 4050)	43.7 (15.4, 172.5)	0.014
	day 4 up	467.3 (76.9, 497)	28.5 (7.6, 206.9)	0.024
TNF-α, pg/mL	day 1 – day 3	1736.3 (411.1, 1877.2)	159.4 (75.2, 567.1)	0.099
	day 4 up	4813.9 (157.5, 23277.8)	143.5 (23.7, 245.3)	0.051
IL1-β, pg/mL	day 1 – day 3	54.4 (47.6, 81.9)	8 (5.4, 19.5)	0.015
	day 4 up	508.9 (29.4, 1017.5)	10.4 (7.1, 20.5)	0.004

4.5 Cytokines for diagnosis of AKI

Figure 10 illustrated the ROC curves of cytokines for diagnosis AKI. In all cases, the area under the ROC curve (AUC) was 0.60 (95% confidence interval (CI) 0.51– 0.69) for IL-6, 0.74 (95% CI 0.66– 0.81) for MCP-1, 0.68 (95% CI 0.59– 0.76) for TNF-α, and 0.69 (95% CI 0.60– 0.78) for IL-1β. (Figure 10) For diagnosis AKI in leptospirosis confirmed cased, the AUC was 0.54 (95% confidence interval (CI) 0.43– 0.65) for IL-6, 0.67 (95% CI 0.56– 0.78) for MCP-1, 0.67 (95% CI 0.57– 0.77) for TNF-α, and 0.60 (95% CI 0.48– 0.71) for IL-1β (Figure 11).

While in non-leptospirosis cases, the area under the ROC curve (AUC) was 0.62 (95% CI 0.43– 0.80) for IL-6, 0.81 (95% CI 0.67– 0.94) for MCP-1, 0.74 (95% CI 0.57– 0.91) for TNF-α, and 0.60 (95% CI 0.48– 0.71) for IL-1β (Figure 12).

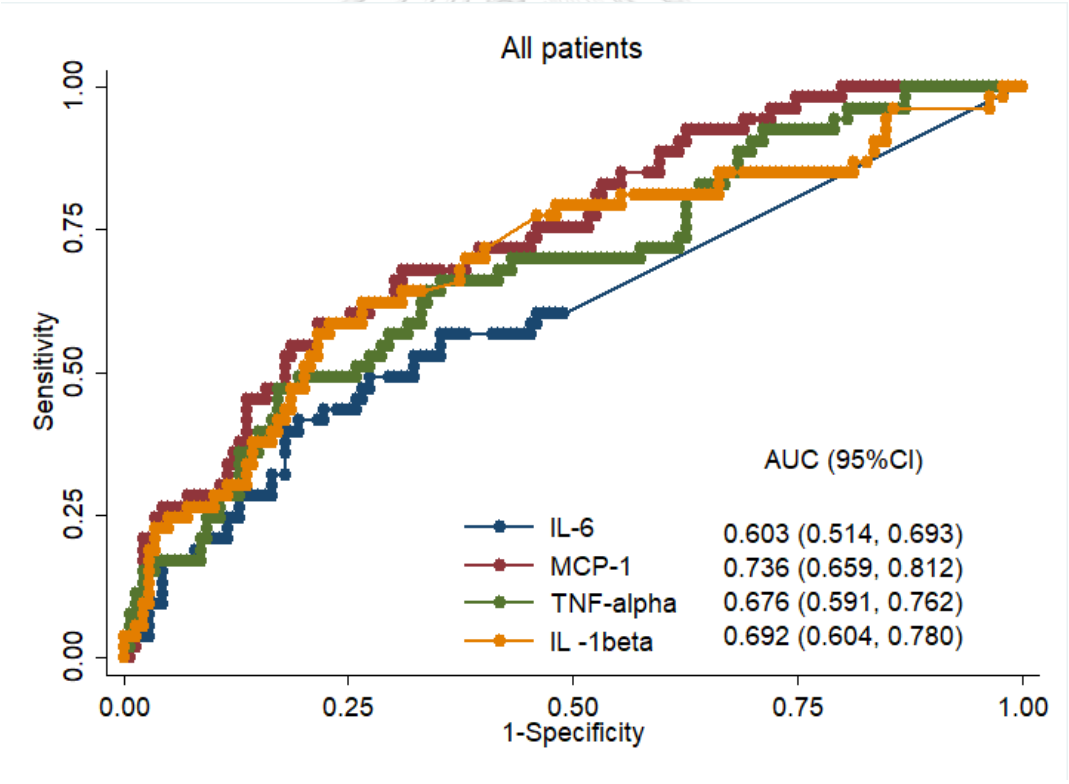


Figure 10. The area under the curve (AUC) for the association between cytokines and AKI in all cases.

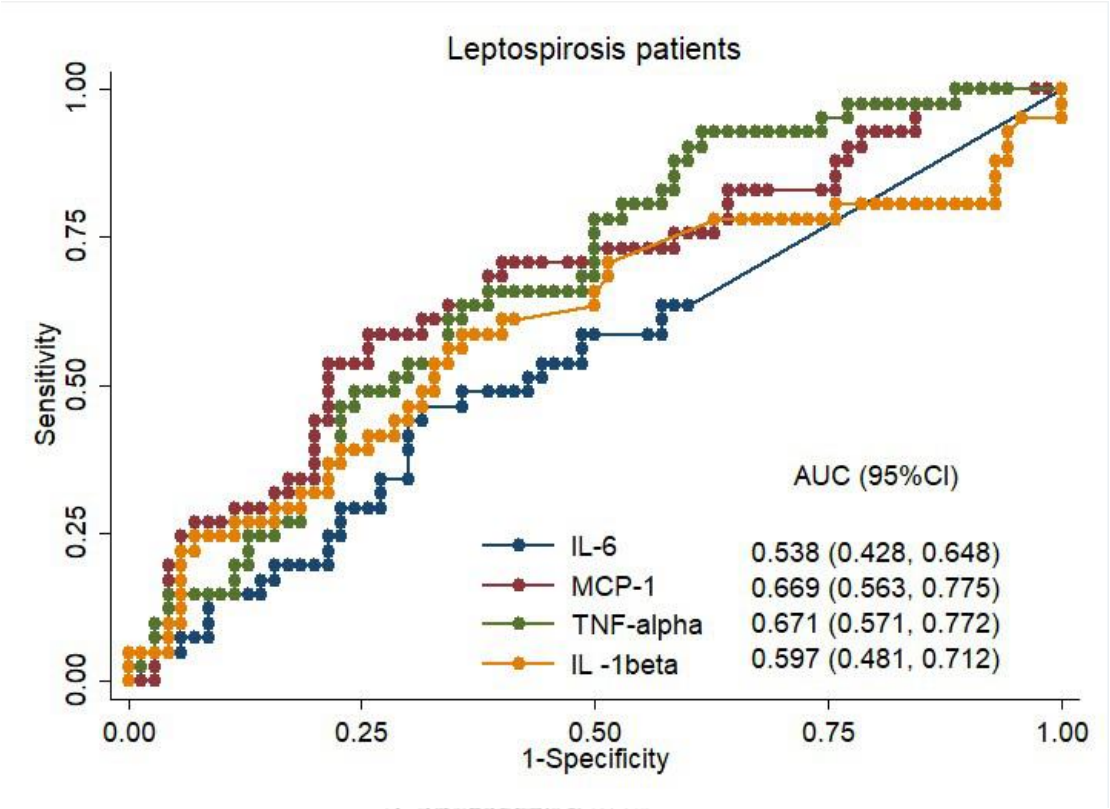


Figure 11. The AUC for the association between cytokines and AKI in leptospirosis confirmed cases.

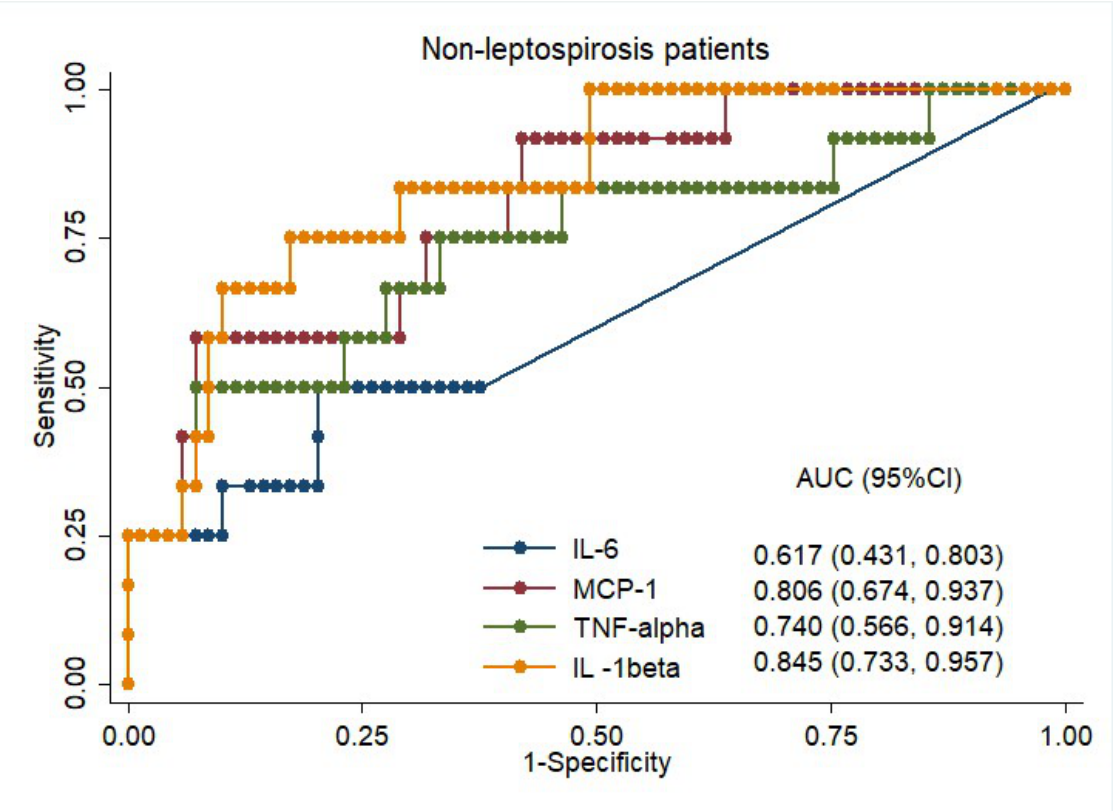


Figure 12. The AUC for the association between cytokines and AKI in non leptospirosis cases.

4.6 Analysis of cytokines associated with AKI

Focusing on the predicting model for AKI in all cases, we found uNGAL, pNGAL, IL-6, body temperature, low platelet count, and bicarbonate independently associated with AKI, odds ratio (OR) 8.58 (95% CI 3.09-23.86), P < 0.001; 1.70 (95% CI 1.32– 2.18), P < 0.001; 0.51 (95% CI 0.26-0.99), P = 0.047; 0.50 (95% CI 0.26-0.88), P = 0.018; 0.90 (95% CI 0.83–0.96), P = 0.001; and 0.90 (95% CI 0.82-0.99), P = 0.043, respectively) (Table 11).

In leptospirosis confirmed cases, the adjusted model still showed uNGAL, pNGAL, IL-6, low platelet count, and bicarbonate independently associated with AKI, odds ratio (OR) 13.25 (95% CI 3.10-56.59); 1.86 (95% CI 1.29– 2.69); P = 0.001; 0.39 (95% CI 0.17-0.89), P = 0.025; 0.37 (95% CI 0.16–0.87), P = 0.023; and 0.85 (95% CI 0.76-0.95), P = 0.006, respectively) (Table 11).

Table 11. Analysis of biomarkers as predictors of AKI (A) in all cases, (B) in leptospirosis cases, and (C) in non-leptospirosis cases

Biomarker	Odds ratio (95%CI) unadjusted	P-value	Odds ratio (95%CI) adjusted[a]	P-value	Odds ratio (95%CI) adjusted[b]	P-value
A. For all patients (N=206)						
uNGAL x 100 ng/ml	2.58 (1.68,3.95)	<0.001	8.58 (3.09,23.86)	<0.001		
pNGAL x 100 ng/ml	1.48 (1.33,1.64)	<0.001			1.70 (1.32,2.18)	<0.001
IL-6 (log scale)	1.24 (1.04, 1.48)	0.016	0.74 (0.44, 1.23)	0.249	0.51 (0.26, 0.99)	0.047
MCP-1 (log scale)	1.68 (1.35, 2.09)	<0.001	1.62 (0.92, 2.88)	0.097	1.87 (0.86, 4.06)	0.115
TNF-α (log scale)	1.36 (1.16, 1.59)	<0.001	1.03 (0.73, 1.44)	0.872	1.12 (0.73, 1.72)	0.597
IL-1β (log scale)	1.50 (1.22, 1.78)	<0.001				
Serum creatinine x 0.1, mg/dl	1.27 (1.17,1.39)	<0.001				
Body temperature, ºc	0.67 (0.51,0.88)	0.005	0.50 (0.26, 0.88)	0.018	0.52 (0.25, 1.05)	0.069
WBC x 5000	1.10 (1.04,1.16)	0.001				
Hgb, g/dl	0.79 (0.69,0.92)	0.002				
Platelet x 10000	0.88 0.84,0.93)	<0.001	0.94 (0.87,1.01)	0.071	0.90(0.83,0.96)	0.001
TB, mg/dl	1.08 (1.01,1.62)	0.024				
DB, mg/dl	1.15 (1.02,1.29)	0.020				
SGOT x 10, u/ L	1.05 (0.99,1.12)	0.088				

SGPT x 10, u/ L	1.01 (1.00,1.02)	0.204				
HCO3, mEq/ L	0.94 (0.89,0.99)	0.013			0.90(0.82,0.99)	0.043
B. For Leptospirosis patients (N=113)						
uNGAL x 100 ng/ml	2.58(1.68,3.95)	<0.001	13.25 (3.1, 56.59)	<0.001		
pNGAL x 100 ng/ml	1.40(1.25,1.58)	<0.001			1.86 (1.29, 2.69)	0.001
IL-6 (log scale)	1.07 (0.88, 1.29)	0.488	0.7 (0.37, 1.31)	0.263	0.39 (0.17, 0.89)	0.025
MCP-1 (log scale)	1.41 (1.09, 1.82)	0.008	1.67 (0.75, 3.69)	0.209	1.86 (0.68, 5.08)	0.229
TNF-α (log scale)	1.30 (1.09, 1.55)	0.004	1.04 (0.68, 1.6)	0.855	1.07 (0.63, 1.82)	0.805
IL-1β (log scale)	1.21 (0.99, 1.51)	0.068				
Serum creatinine x 0.1, mg/dl	1.20(1.11,1.30)	<0.001				
Body temperature, ºc	1.12(1.03,1.21)	0.006	0.37 (0.16, 0.87)	0.023	0.55 (0.23, 1.32)	0.180
WBC x 5000	0.81(0.68,0.97)	0.023				
Hgb, g/dl	0.91(0.86.0.96)	0.001				
Platelet x 10000			0.91 (0.82, 1.00)	0.057	0.85 (0.76, 0.95)	0.006
TB, mg/dl	1.07(1.00,1.16)	0.064				
DB, mg/dl	1.16(1.00,1.34)	0.044				
SGOT x 10, u/ L	1.02(0.96,1.08)	0.575				
SGPT x 10, u/ L	1.00(0.99,1.02)	0.786				
HCO3, mEq/ L	1.20(1.11,1.30)	<0.001			0.90 (0.70, 1.14)	0.371
C. For Non-leptospirosis patients (N=93)						
uNGAL x 100 ng/ml	2.11(0.99,4.48)	0.051	4.62 (0.55, 38.89)	0.160		
pNGAL x 100 ng/ml	1.58(1.25,2.02)	<0.001			2.41 (0.64, 9.01)	0.193
IL-6 (log scale)	1.68 (1.09, 2.61)	0.020	0.65 (0.08, 5.08)	0.678	2.83 (0.31, 25.65)	0.355
MCP-1 (log scale)	2.17 (1.35, 3.48)	0.001	1.51 (0.6, 3.84)	0.384	0.46 (0.06, 3.89)	0.479
TNF-α (log scale)	1.64 (1.15, 2.34)	0.006	1.04 (0.45, 2.41)	0.921	1.44 (0.59, 3.49)	0.419
IL-1β (log scale)	2.21 (1.42, 3.45)	<0.001				
Serum creatinine x 0.1, mg/dl	3.2(1.01,10.2)	0.049				
Body temperature, ºc	0.19(0.42,1.04)	0.073	0.85 (0.27, 2.7)	0.786	0.25 (0.03, 2.03)	0.195
WBC x 5000	0.98(0.93,1.03)	0.379				
Hgb, g/dl	0.9(0.83,0.98)	0.012				

Platelet x 10000	1.02(0.89,1.22)	0.472	0.99 (0.87, 1.12)	0.834	0.88 (0.7, 1.11)	0.282
TB, mg/dl	1.02(0.85,1.33)	0.180				
DB, mg/dl	1.02(0.99,1.06)	0.112				
SGOT x 10, u/ L	0.18(0.48,0.81)	<0.001				
SGPT x 10, u/ L	3.2(1.01,10.2)	0.049				
HCO3, mEq/ L	0.19(0.42,1.04)	0.073			0.54 (0.18, 1.58)	0.261

[a] adjusted model for uNGAL, [b] adjusted model for pNGAL

4.7 Quantification of Leptospiral burden and AKI

We used the second cohort to test the role of leptospiral burden and Aki in leptospirosis infection.

In the second cohort, of the 330 subjects with clinical suspicion of leptospirosis (all cases), urine samples were unavailable in 6. These cases and 28 additional patients that could not be identified for AKI status were excluded. Of the remaining 296, 207 patients were confirmed the diagnosis of leptospirosis with 62 patients with AKI and 149 patients without AKI (Figure 13).

The leptospiral load on the first day of enrollment in AKI group was 870 leptospira/mL while in non-AKI was 402 leptospira/mL, P = 0.21. On day 7 after enrollment. There was no difference of leptospiral load in AKI and non-AKI (Figure 14). We also explored the association of leptospiral load and severity of AKI. On the first day of enrollment, severe AKI (defined as AKI stage 3) had leptospiral load higher than non-severe AKI (defined as non-AKI, AKI stage 1, and 2), 1459 (IQR 24307) vs 385 (IQR 1832) leptospira/mL, P = 0.004. However, there was no difference of leptospiral load between severe AKI and non-severe AKI on day 7 after enrollment (Figure 15).

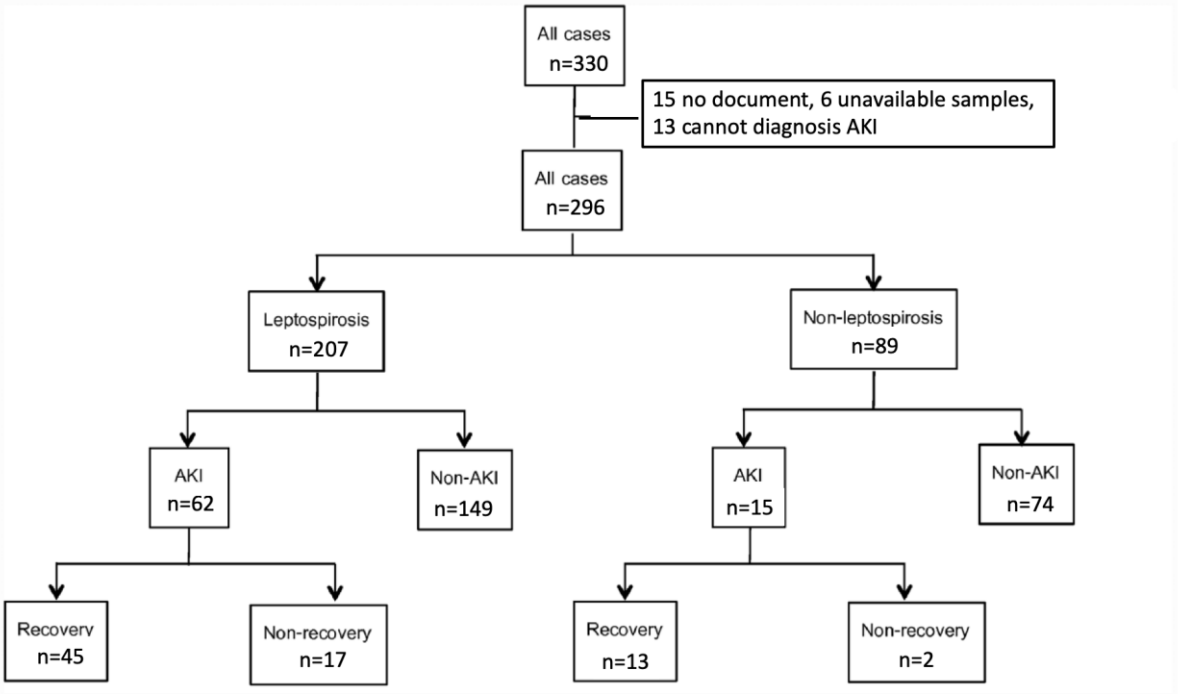


Figure **13.**Subject disposition for the second Thai lepto AKI study cohort

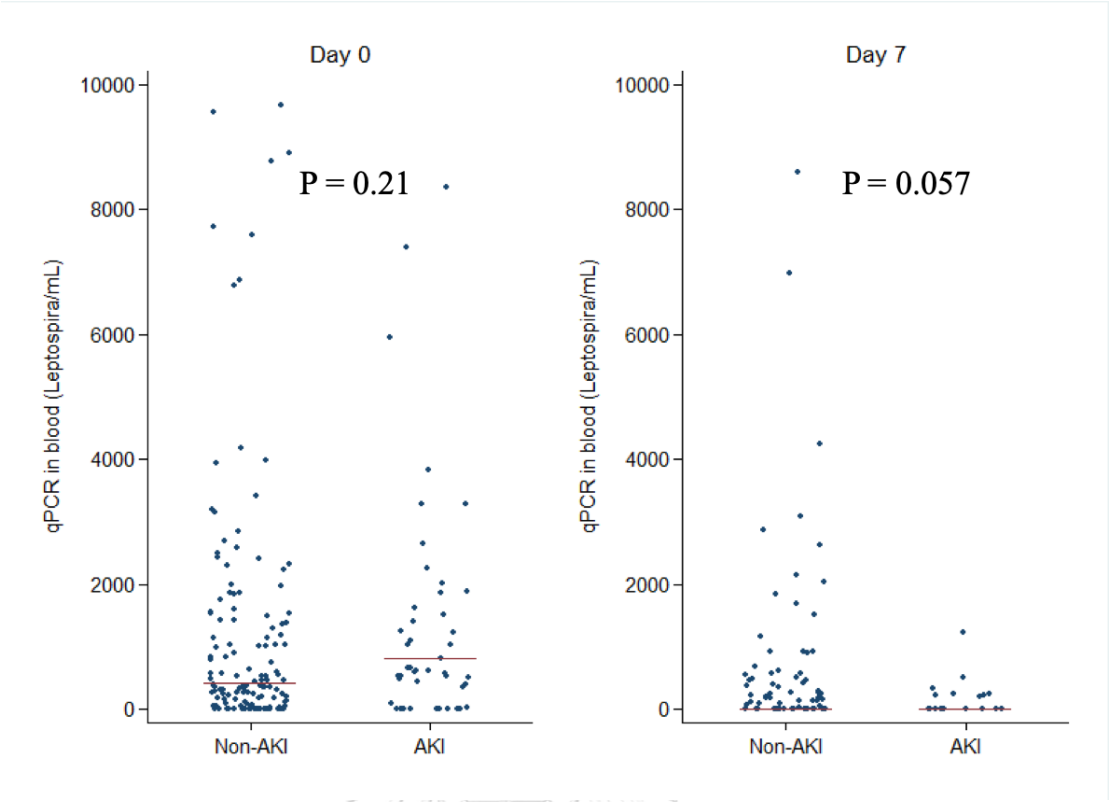


Figure 14. Real-time PCR in blood between AKI and non-AKI group among leptospirosis patients on day 1 (N=207) and day 7 after enrollment (N=92).

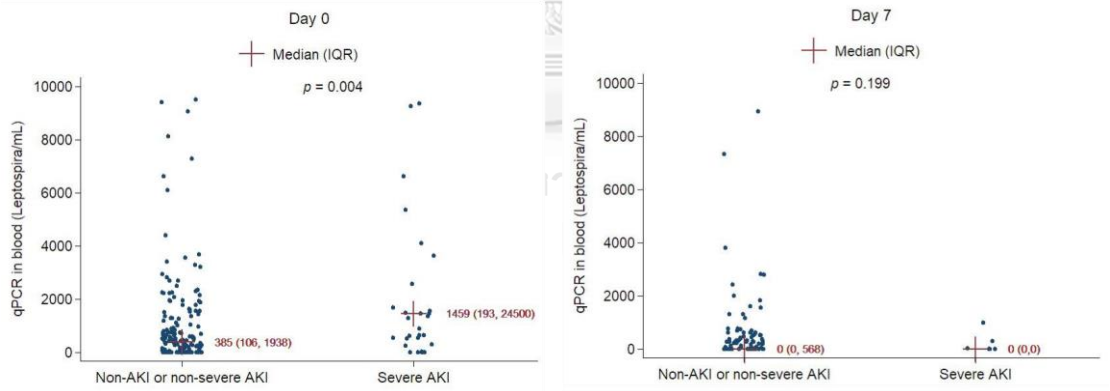


Figure 15. Real-time PCR in blood between severe AKI and non-severe AKI in leptospirosis patients on day 1 (N=207) and day 7 after enrollment (N=92).

The AUC of leptospiral load in diagnosis AKI was 0.56 (95%CI 0.46-0.65), while AUC for prediction severe AKI was 0.65 (95%CI 0.54-0.76). At the cutoff-point 756.7 of leptospiral load showed the sensitivity, specificity, accuracy, positive likelihood ratio, and negative likelihood ratio of 63.2%, 59.2%, 59.9, 1.55, and 0.62, respectively (Figure 16).

We also showed leptospiral load stratified by day of fever between AKI and non-AKI patients. On the first 3 day, AKI patients had significant higher leptospiral load than non-AKI patients, AKI group VS non-AKI group 869.7(95%CI 165.6-18965.9), vs 393.8 (95%CI 121.7-1958.5), P = 0.057. There was no difference of leptospiral load after day 3 between AKI and non-AKI patients (Figure 17).

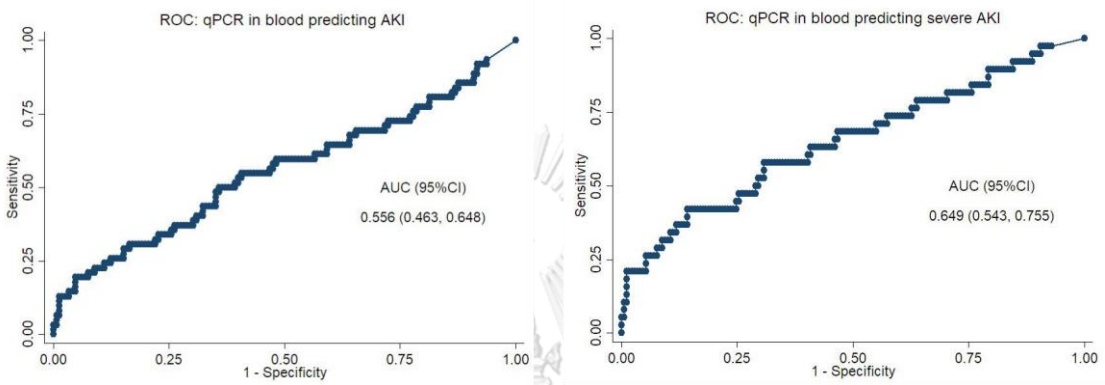


Figure 16. The AUC for the association between quantity of leptospires and AKI status (left figure) and severity of AKI (right graph)

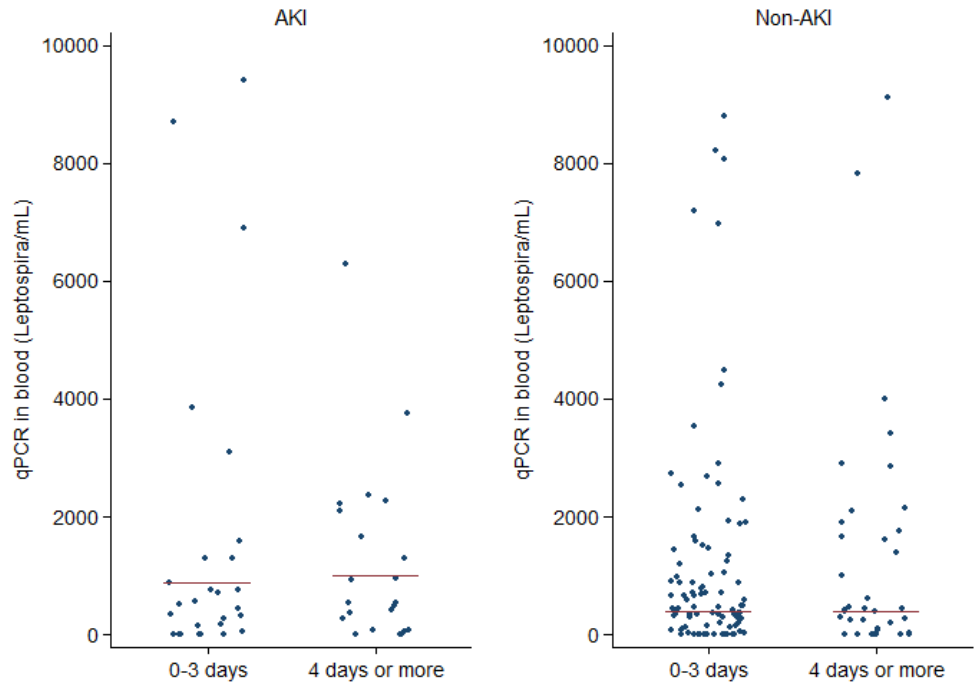


Figure 17.Real-time PCR in blood stratified by day of fever between AKI and non-AKI group.

4.8 Leptospira serovar in AKI

We also explored the association of leptospira serovar and AKI in leptospirosis infectin. The species which were found the most was Shermani (29/51) then Australis (11/65). When we stratified by AKI status, we found 11 (73%) Shermani in AKI patients (N=15) and 18 (50%) Shermani in Non-AKI patients (N=36) but there was no statistical difference in proportion of this specie between AKI and non-AKI patients and for other species (Figure 18).

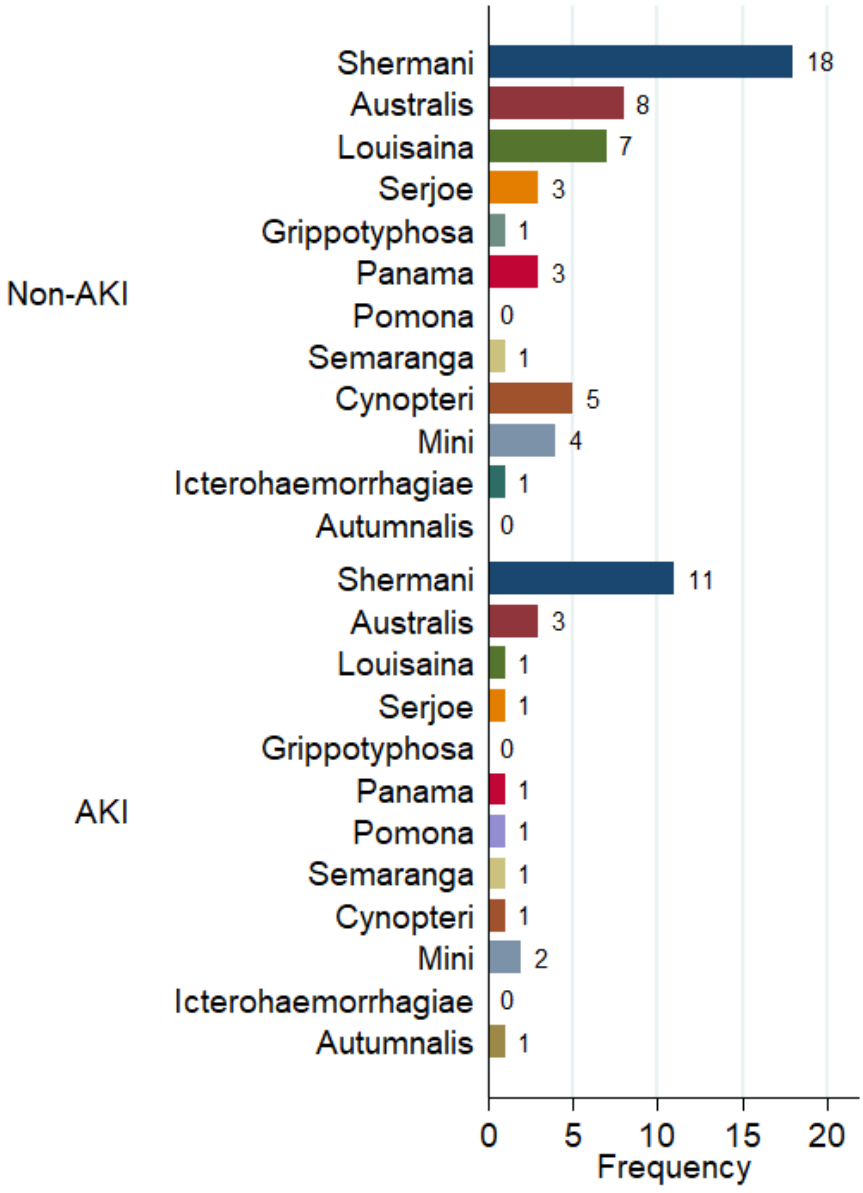


Figure 18. Leptospira serovar stratified by AKI status

4.9 Leptospira species and the AKI status

In leptospirosis cohort 2, we also demonstrate leptospira species stratified by AKI status in Table 12. Leptospira interrogans was the most common species which was discovered.

Table 12. Leptospria species stratified by AKI status

Leptospiral species	Non-AKI	AKI stage 1	AKI stage 2	AKI stage 3	Total
L. interrogans	9	1	2	0	12
L. kirschneri	1	0	0	0	1
	10	1	2	0	13

4.10 Association between antibody titer and AKI status in leptospirosis

We also explored the association of the degree of immune response (defined as the MAT titer) and AKI status in both cohort 1 (MAT positive N = 65 cases), and 2 (MAT positive N = 51. In the first cohort, AKI patients had higher MAT titer than non-AKI patients, 3200 (1600,6400) vs 1600 (400,6400), P = 0.040. However, we did not find any difference of MAT titer between AKI and non-AKI cases in the second cohort (Figure 19). When we combined the first and second cohort together (n=126 cases), we did not find any difference of MAT titer between AKI and non-AKI cases (Figure 20). However, when we focusing on severe AKI, we found severe AKI cases had significantly higher MAT titer than non-severe AKI cases (Figure 21).

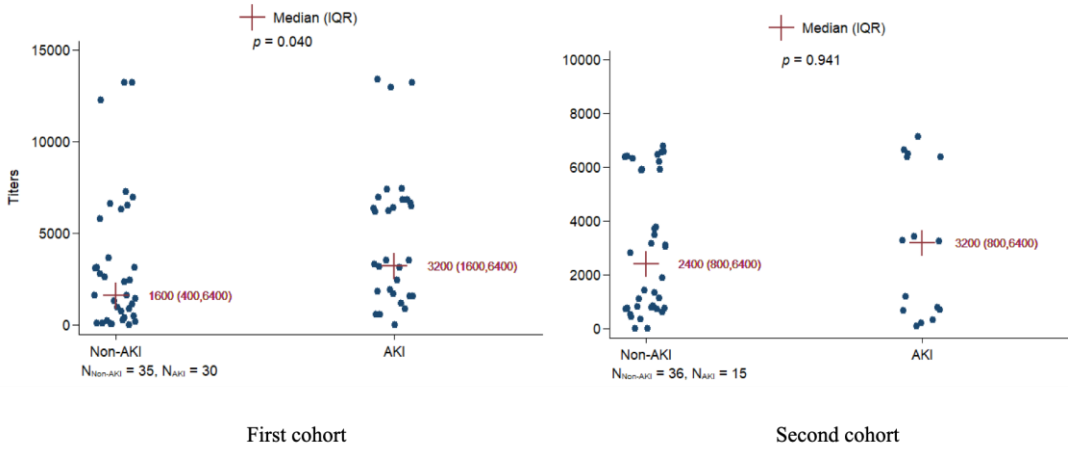


Figure 19. MAT titers stratified by AKI status in cohort 1 and 2.

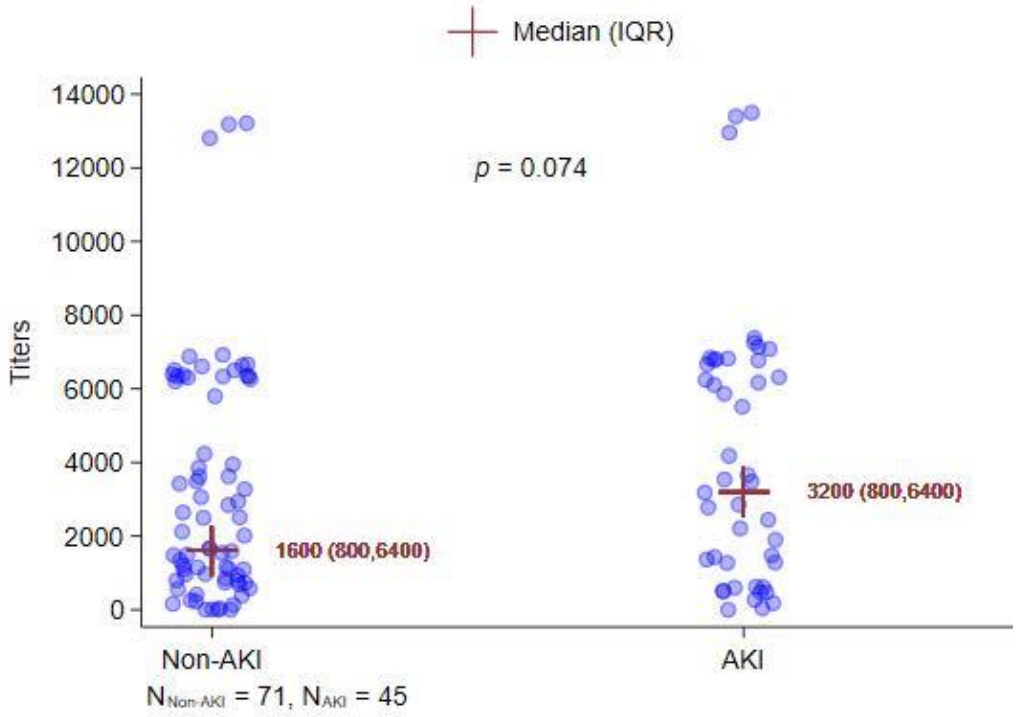


Figure 20. MAT titers stratified by AKI status in combined cohort

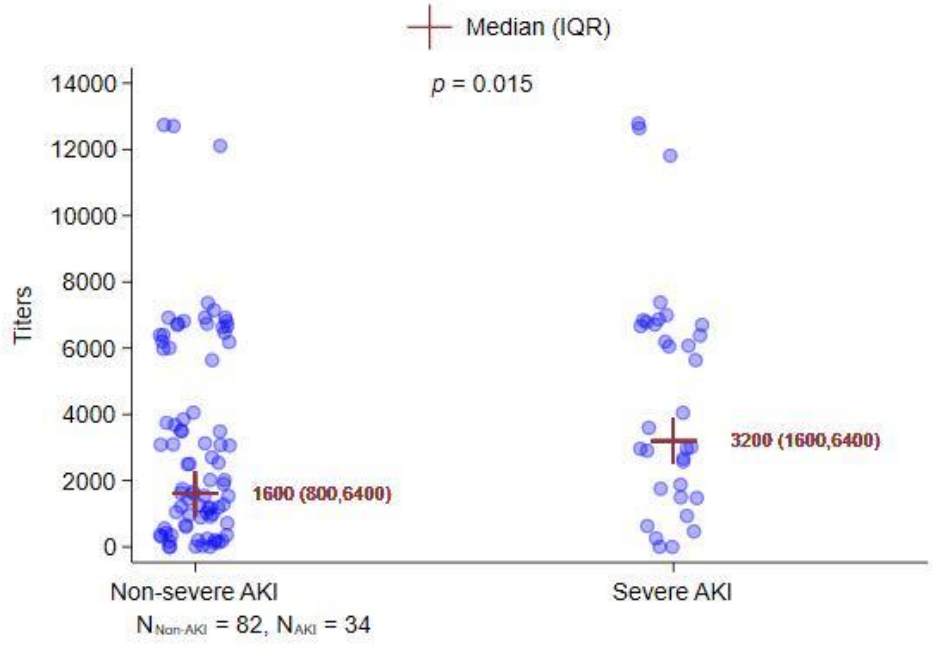


Figure 21. MAT titer stratified by severity of AKI in combined cohort

CHAPTER 5: DISCUSSION

In this study, we explore the role of host immune response and pathogen (represented by cytokines, leptospiral burden, leptospiral species, leptospiral serovar, and anti-leptospira agglutinating antibodies) in human leptospirosis associated AKI. This study should be the first step to provide the better understanding of the host pathogen interaction. The data and analysis here allow us to describe several important findings. We found that MCP-1, TNF-α, and IL-1β concentrations on the first day of enrollment were significantly higher in leptospirosis confirmed cases who developed AKI. When stratified cytokines level by day of fever, only MCP-1 level after day 3 of fever and TNF-α on the first 3 day of fever were significantly higher in leptospirosis confirmed cases who developed AKI. MCP-1 and TNF-α showed the highest AUC in the diagnosis of AKI. Moreover, only low IL-6 showed strong association with AKI by a covariate-adjusted model. On the first day of enrollment, severe AKI had leptospiral load higher than non-severe AKI with the AUC for prediction severe AKI was 0.65. At the cutoff-point 756.7 of leptospira/mL showed the sensitivity, specificity, accuracy, positive likelihood ratio, and negative likelihood ratio of 63.2%, 59.2%, 59.9, 1.55, and 0.62, respectively. Finally, we quantified anti-leptospira agglutinating antibodies titer and AKI status, and found high antibodies titer associated with severe AKI.

AKI can be a diagnostic challenge in patients with leptospirosis because AKI is one of the most serious complication of leptospirosis infection. Current maker, serum creatinine may be interfered by many factors such as volume overload, rhabdomyolysis, and jaundice. These confounding factors together make serum creatinine less sensitive and specific in the setting of leptospirosis. Most of leptospirosis patients live in rural areas and shortages of nephrologists and dialysis equipment are common. Study pathogenesis of leptospirosis associated AKI would fill the missing gap of knowledge and identify the potential treatment to improve outcome of leptospirosis.

Previous has explored the role of cytokines/chemokines in severe leptospirosis, and found the conflicting results (28-33). Unfortunately, no study has been specifically designed to study the role of cytokines in leptospirosis associated AKI. We discovered that MCP-1, TNF-α, and IL-1β had the potential role to be the

markers of AKI in leptospirosis (Figure 2,3,4 and Table 3). Possible explanations on the discrepancies among the study of cytokines on severe leptospirosis could be due to many factors. Firstly, the time of cytokines measurement of each study was various and was tested at the different stages of infection. Second, we are still lack of the standard definition of severe leptospirosis. Third, the technique to test cytokines was different such as standard ELISA, or using Luminex assay. Fourth, the gold standard to confirm leptospirosis is different. Some studies used the combination of microscopic agglutination (MAT), culture and polymerase chain reaction (PCR), some studies used only MAT, and some studies used the combination of PCR, MAT, etc. Fifth, most of the studies have the small number of sample size, and Lastly, most of the studies did not use the multivariate analysis to adjust the confounding factors. Chirathaworn et al proposed the idea that delayed and sustained pro-inflammatory cytokines production, high levels of pro-inflammatory mediators, and delayed anti-inflammatory cytokine production could lead to persistence or delay of bacteria clearance and severe tissue/organ damage (21). In light of the important of cytokines in the pathogenesis of leptospirosis associated AKI, removal cytokines could be one of the potential treatment to improve outcome. Currently, there are many columns (cartridge) in blood purification which are designed to adsorb cytokines at the surface membrane (69). However, cytokine reduction have been correlated with reduction in vasopressor use, improvement of mean arterial pressure and severity score, the survival benefit is still need to be more validated (70-72).

Previous studies showed the conflicting results of the predictive role of high bacteremia (leptospiral load) and disease severity (23, 24, 73). Our study demonstrated that high bacterial load could have a role in prediction AKI severity. This finding provides the new role of real-time PCR which is not only the standard diagnostic test of leptospirosis but also the new severity marker for AKI. Previous studies proposed the critical threshold of 10^3leptospires/mL as the cutoff point which correlate disease severity. This number is closely to our study which found leptospires at 800 leptospries/mL correlate with severe AKI. To our knowledge, our study is the first one that clearly delineates as association between leptospiral burden and severe AKI. Early bacterial load determination could provide an alert to physicians in their management of high risk patients

We also explored the role of specific antibody response to leptospira and the severity of AKI. This is the first time which we can demonstrate high antibody response associate with severe AKI (Figure 17). In contrast to previous report by Lindow JC et al, this study showed that high antibody response related to better survival (74). We hypothesize that the optimal amount of antibody response is the crucial in neutralized the effect of leptospiral antigen. However, if the amount of antibody is too high to the neutralization effect, this can do harm to the organ (75). Another possible hypothesis was the non-specificity of antibody response to leptospiral antigen. This may explain why high antibody response cannot neutralize the effect of leptospiral antigen and the discrepancy finding between our finding and previous study.

There are several limitations to our study. First, due to the study design and variable timing of hospital admission, we cannot test all patients from the first day of fever. Therefore, most of our patients had increased serum creatinine since the first day of enrollment. Therefore, the interpretation of the result for the role of cytokines/chemokines, leptospiral load and antibody response in association with AKI need to understand this limitation. However, we have tried to reduce this bias by stratification our patients by day of fever. After adjust this factor, we still found that MCP-1 and TNF-α were significantly higher in patients with AKI than in patients without. Second, we have measured biomarkers at only two time point (on the first day of enrollment) and could not assess the value of cytokines as repeated measures. Nevertheless, risk stratification and prognostication using a biomarker is likely to be useful only when biomarker concentrations are measured early. Third, we were unable to measure process of care variables such as the effect of co-interventions on cytokines level and its influence on development of AKI. Fourth, we found that cytokines did not have any association with renal recovery. Our finding should be interpreted with caution because the limitation number of patients in our study. Only 10 patients from 55 AKI patients did not recover.

Our study has several strengths. First, the study design is a multicenter study testing biomarkers in hospitalized patients with leptospirosis, therefore the results are highly applicable to this specific population. Second, we chose the first day of presentation to medical attention (the first day of clinical suspicious leptospirosis) as

the time to test cytokines and leptospiremia. This time point was match to real clinical situation. Because most of leptospirosis patients came to hospital on various day of fever, so it was hard to test biomarkers only on the first day of fever. Third, we have serially tested cytokines level in two times points which will allow us to explore the dynamic of cytokines and the association with AKI. Fourth, we created the multivariate model adjusted model to explore the association of cytokines and AKI in leptospirosis. This model will reduce the confounding from the other factors which could affect the cytokines level.

In summary, MCP-1, TNF- α appears to be useful markers for detecting AKI in Leptospirosis patients. In the adjusted model for severity, only low IL-6 is associated with AKI. Our data suggest that high leptospiremia and high anti-leptospira antibody correlate with the severity of AKI.

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