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ORIGINAL ARTICLE

Raised levels of Il-6, Il-17a, and Il-22 in fatal leptospirosis

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Abstract

Clinical manifestations of leptospirosis range from mild, common cold-like illness, to a life-threatening condition. The host immune response has been hypothesized to play a major role in leptospirosis outcome. Increased levels of inflammatory mediators, such as cytokines, may promote tissue damage that lead to increased disease severity. The question is whether cytokines levels may predict the outcome of leptospirosis and guide patient management. This study aimed to assess the association between Th1-, Th2-, and Th17-related cytokines with the clinical outcome of patients with leptospirosis. Different cytokine levels were measured in fifty-two plasma samples of hospitalized patients diagnosed with leptospirosis in Malaysia (January 2016–December 2017). Patients were divided into two separate categories: survived ($n = 40$) and fatal outcome ($n = 12$). Nineteen plasma samples from healthy individuals were obtained as controls. Cytokine quantification was performed using Simple Plex™ assays from ProteinSimple (San Jose, CA, USA). Measurements were done in triplicate and statistical analysis was performed using GraphPad software and SPSS v20. IL-6 ($p = 0.033$), IL-17A ($p = 0.022$), and IL-22 ($p = 0.046$) were significantly elevated in fatal cases. IL-17A concentration (OR 1.115; 95% CI 1.010–1.231) appeared to be an independent predictor of fatality of leptospirosis. Significantly higher levels of TNF-α ($p \leq 0.0001$), IL-6 ($p \leq 0.0001$), IL-10 ($p \leq 0.0001$), IL-12 ($p \leq 0.0001$), IL17A ($p \leq 0.0001$), and IL-18 ($p \leq 0.0001$) were observed among leptospirosis patients in comparison with healthy controls. Our study shows that certain cytokine levels may serve as possible prognostic biomarkers in leptospirosis patients.

Keywords Cytokines · Leptospirosis · Clinical outcome · Biomarkers · T helper cell · Malaysia

Introduction

Differences in cytokine patterns due to the host immune response during the course of *Leptospira* infection have been proposed as factors for the variation in disease severity [1, 2]. It was demonstrated that several *Leptospira* components are capable of inducing pro-inflammatory cytokines. Hemolysin, a toxin secreted by pathogenic *Leptospira interrogans*, for instance, was found to stimulate the productions of pro-inflammatory cytokines in human cells by inducing both Toll-like receptors 2 (TLR2) and TLR4-dependent nuclear factor (NF)-κB and c-Jun N-terminal kinase (JNK) pathways

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[3, 4]. Leptospiral lipopolysaccharide (LPS) was shown to stimulate macrophages via the TLR2 pathways as well [5, 6].

This was later supported by clinical studies demonstrating cytokine overexpression during acute *Leptospira* infection, also encompassing T helper type 1 (Th1), T helper type 2 (Th2), and T helper type 17 (Th17) responses [7]. Different expression patterns of cytokines correlated with clinical outcome [8, 9]. Cytokines proposed as markers for severity include tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-10 [9, 10]. TNF-α, a pro-inflammatory cytokine expressed by Th1 helper cells upon activation by antigen presenting cells (APC), was significantly higher in severe cases as compared with fatal cases of leptospirosis [9, 11, 12]. IL-10, one of the major cytokines expressed by Th2 cells, was significantly higher in severe and fatal disease as compared with mild disease [9, 10, 13]. Most previous studies show IL-6 as being significantly higher expressed in severe cases than in mild leptospirosis [7, 9, 13].

The aim of the present study was to determine the association of Th1-related (IL-12, IL-18, and TNF-α), Th2-related (IL-4, IL-10, and IL-13), and Th17-related (IL-6, IL-17A, and IL-22) cytokines with the clinical outcomes of leptospirosis patients.

Methods

Study design

This is an observational case-control study of leptospirosis patients hospitalized in Selangor and Perak states of Malaysia from January 2016 to December 2017. Ethical approval for the study was obtained from the Medical Research and Ethics Committee, Ministry of Health Malaysia (NMRR-15-2148-27536 and NMRR-15-756-25320). Samples were collected from patients suspected of leptospirosis after informed consent. Leptospirosis was confirmed by PCR and microscopic agglutination testing (MAT), according to the criteria provided in the World Health Organization (WHO) guidelines [14]. Patients aged less than 18 years and those with diagnoses other than leptospirosis were excluded. Nineteen matched healthy controls were recruited with exclusion criteria of smoking, comorbid/underlying disease, genetic/autoimmune disease, fever/flu in the last 2 weeks, and pregnancy.

Study samples

Venipunctures were performed on the medial cubital or cephalic vein by a certified phlebotomist or medical doctor. Six milliliters of blood collected in ethylenediaminetetraacetic acid (EDTA) tubes was centrifuged and plasma was separated, aliquoted, and stored at − 80 °C at the day of collection.

Demography and clinical data collection

Patient demographics, medical background, and clinical data were documented. Patients were divided into two groups: those that survived ($n = 40$) and those with a fatal outcome ($n = 12$). Severe organ involvement was defined through laboratory analyses; liver dysfunction was described with jaundice and/or total bilirubin of > 20.5 μmol/L, blood alanine aminotransferase (ALT) of > 55 U/L, blood aspartate aminotransferase (AST) of > 34 U/L. Renal dysfunction was described as oliguria (passing urine less than 0.3 mL/kg/day) and/or abnormal creatinine level of > 115 μmol/L and blood urea nitrogen (BUN) of > 9.2 mmol/L. Pulmonary dysfunction was described in case of pneumonia, hemoptysis, and/or abnormal chest radiography or required mechanical ventilation support. Hemorrhagic manifestations were described in case of bleeding. These classifications were adapted with minor modifications from a previous publication [13].

Simple Plex assays for cytokines

Concentrations of TNF-α, IL-4, IL-6, IL-10, IL-12, IL-13, IL-17A, IL-18, and IL-22 were measured in triplicate using Simple Plex™ assays from ProteinSimple (San Jose, CA, USA), and expressed in picogram per milliliter. Briefly, plasma samples were diluted 1:2 by mixing 35 μL of sample with 35 μL sample diluent, following the manufacturer's instructions [15]. Then, 1000 μL of wash buffer and 50 μL of diluted plasma samples were loaded via the inlets on the test cartridge. The loaded test cartridges were analyzed using an Ella analyzer (San Jose, CA, USA).

Statistical analysis

Statistical analyses were performed using GraphPad® Prism v6 software (San Diego, CA, USA) and SPSS v20 (Chicago, IL, USA). Descriptive statistics are presented as median values with a range for continuous data and as number and percentages for categorical data. Mann-Whitney *U* tests were applied to evaluate the significance of the differences in cytokine levels between groups. Multiple comparison tests (Kruskal-Wallis with Dunn's multiple comparison for post hoc analysis) were used to determine the statistical significance of differences in cytokine expression between groups. Statistically significant variables ($p \leq 0.05$) in a univariate test were selected to be included in the multivariate logistic regression analysis (stepwise backward) to determine the inter-relationships between the cytokines.

Results

Leptospirosis patient characteristics

Fifty-two adult patients with laboratory confirmed diagnosis of leptospirosis were recruited (Table 1). Among these 52 patients, 12 (23%) were associated with fatal outcome while 40 (77%) suffered mild and severe disease but survived. The majority consisted of male patients. Patients with fatal outcome had the highest median age. Laboratory investigation reveals that fatal cases exhibit higher levels of BUN ($p \leq 0.019$) and serum creatinine ($p \leq 0.018$) when compared with surviving patients.

Cytokine concentrations and severity of disease

Of the nine cytokines evaluated, the expressions of TNF-α ($p \leq 0.0001$), IL-6 ($p \leq 0.0001$), IL-10 ($p \leq 0.0001$), IL-12 ($p \leq 0.0001$), IL17A ($p \leq 0.0001$), and IL-18 ($p \leq 0.0001$) were found significantly higher among leptospirosis patients (survived and fatal combined) as compared with the healthy controls. The expressions of IL-6 ($p \leq 0.0325$), IL-17A ($p = 0.0219$), and IL-22 ($p = 0.0459$) were found significantly higher among fatal as compared with the survived cases of leptospirosis (Table 2). These three statistically significant differences in cytokine concentrations between fatal and surviving cases were included in multivariate logistic regression analysis (stepwise backward) to determine the interrelationships between the cytokines (Table 3). Higher concentrations of IL-17A (odds ratio (OR) 1.115; 95% confident intervals (CI) 1.010–1.231) were independently associated with the fatality of leptospirosis among hospitalized patients.

Discussion

Our study found that Th17-related IL-6, IL-17A, and IL-22 levels were significantly higher among fatal leptospirosis cases compared with survivors. In a previous study, Reis et al. had found that IL-17A expression was significantly higher in severe than in mild cases of leptospirosis [9]. Current findings, however, do not correspond with certain prior reports. Recent data from Bandara et al. and Papa and Kotrotsiou failed to correlate IL-17A levels between healthy controls, mild cases, and severe cases of leptospirosis [7, 16]. Nonetheless, this current study demonstrated a significantly higher IL-6 expression ($p = 0.0190$) in fatal cases, when compared with mild cases of leptospirosis. This is in agreement with the data achieved by Chirathaworn et al. and Reis et al., in which high levels of IL-6 also correlated with organ dysfunction and SPHS, respectively [9, 13]. Interestingly, previous studies had also shown that IL-6 expression was found to be markedly high only during the early stages of severe cases, prior to reaching their normal level again [7]. To the best of our knowledge, we present the first study including IL-22 in the association of cytokine levels with clinical outcome of leptospirosis. Together, these findings suggest a possible role of IL-6, IL-17A, and IL-22 as potential prognostic biomarkers in evaluating and predicting leptospirosis clinical outcome.

Table 1 Characteristics of demographic, clinical presentation, and laboratory investigation of included leptospirosis patients

Variable	Survived ($N = 40$)	Fatal ($N = 12$)
Demographic		
Average age (range)	35 (18–77)	64 (33–83)
Male (%)	29 (73)	6 (50)
Clinical presentation		
Fever (≥ 38 °C) (%)	40 (100)	12 (100)
Lung involvement (%)	4 (10)	1 (8)
Renal involvement (%)	11 (28)	4 (33)
Liver involvement (%)	12 (30)	3 (25)
Renal and liver involvement (%)	6 (15)	8 (67)
Bleeding (%)	NA	1 (8)
Lab investigations		
BUN* (RR 3.2–9.2) mmol/L	5.3 (1.8–40.4)	15.8 (8.0–22.2)
Serum creatinine* (RR 62–115) μmol/L	82 (46–729)	229 (118–396)
AST (RR 5–34) U/L	43 (13–436)	61.0 (33–688)
ALT (RR 0–55) U/L	35.5 (8–265)	38.0 (14–659)
Bilirubin (RR 3.4–20.5) μmol/L	12.7 (2.7–89.9)	16.3 (6.2–467)

N, number; *RR*, reference range; *NA*, not available

*$p < 0.05$

Table 2 Plasma cytokine levels (pg/mL) in healthy controls and leptospirosis patients (Mann-Whitney *U* test)

	Healthy controls Median (range)	Survived	Fatal	*p* value survived-control	*p* value fatal-control	*p* value survived-fatal
TNF-α	6.120 (2.840–10.07)	18.24 (8.22–106.4)	35.42 (16.47–321.6)	< 0.0001*	< 0.0001*	NS
IL-4	0.0 (0.0–0.4)	0.0 (0.0–0.38)	0.03 (0.0–13.82)	NS	NS	NS
IL-6	2.23 (0.62–4.24)	51.49 (1.65–622.4)	214.0 (43.62–417,012)	< 0.0001*	< 0.0001*	0.0325*
IL-10	2.340 (1.060–3.65)	9.15 (2.14–194.7)	40.66 (10.28–301.9)	< 0.0001*	< 0.0001*	NS
IL-12	6.12 (2.84–10.07)	17.6 (8.22–106.4)	35.42 (16.47–321.6)	< 0.0001*	< 0.0001*	NS
IL-13	1.350 (0.0–3.80)	3.69 (0.02–93)	2.92 (2.92 (0.43–25.82)	NS	NS	NS
IL-17A	1.660 (0.64–4.07)	3.55 (0.05–34.43)	6.805 (3.34–55.44)	0.0041*	< 0.0001*	0.0219*
IL-18	178.7 (64.82–341.1)	844.4 (132.2–51,203)	1278 (229.5–17,926)	< 0.0001*	< 0.0001*	NS
IL-22	22.49 (12.37–47.26)	26.18 (0.25–708.2)	96.76 (11.83–2622)	NS	0.0250*	0.0457*

NS, not significant

*$p < 0.0001$

The current study demonstrated that TNF-α expression was significantly higher among leptospirosis patients when compared with the healthy control group. These findings are consistent with other reports [9, 11, 12]. In a paper published by Rizki et al. on the other hand, the expression of TNF-α was found to be markedly lower in the majority of leptospiral hepatitis patients, when compared with healthy individuals [17]. Correspondingly, Kykiridis et al. had demonstrated a significantly lower TNF-α level in fatal cases of leptospirosis as compared with surviving patients [10]. Our study found that IL-12 and IL-18 were significantly higher among leptospirosis patients compared with the healthy group. However, both cytokines showed no significant association between the differences in patient clinical outcome. This is supported by similar studies done by Reis et al. and Papa and Kotrotsiou, in which no significant differences were observed on IL-12 expression between mild, severe, and fatal cases of leptospirosis [7, 9]. No reports on the association between IL-18 expression and the prognosis of leptospirosis-positive patients have been reported thus far. The present study is among the first to investigate IL-18 as a potential severity marker.

From the three Th2-related cytokines evaluated, only IL-10 was found to be significantly higher in the leptospirosis patients when compared with the healthy control group. Similar observations by Kyriakidis et al. and Chirathaworn et al. show a significantly higher level of IL-10 in severe and fatal disease as compared with mild cases of leptospirosis [10, 13]. A study by Reis et al. shows a significantly higher level of IL-10 among leptospirosis patients with SPHS compared with those without SPHS [9]. In contrast, high levels of IL-10 were observed in mild compared with severe leptospirosis cases [18]. Previous studies including a recently published paper by Jumat et al. in 2018 exhibit no significant differences between the leptospirosis cases and healthy controls [8, 19]. As for IL-4 and IL-13, no significant differences were observed in all severity groups. Similar findings were observed in the study by Papa and Kotrotsiou, which found no significant differences between IL-4 level in healthy controls and mild and severe leptospirosis cases [7]. In contrast, a study by Reis et al. found a significantly higher level of serum IL-4 in severe compared with mild leptospirosis cases [9]. There are no reports on IL-13 association with leptospirosis. Nonetheless, not all Th2-related cytokines did demonstrate significant differences among fatal leptospirosis compared with the survivors.

Table 3 Multivariate logistic regression analysis for prediction of fatality in leptospirosis

	OR	95% CI	*p* value
IL-6	1.00	1.00–1.00	0.977
IL-17A	1.115	1.010–1.231	0.031
IL-22	1.003	0.997–1.008	0.330

The current study is not without limitations. Findings must be further strengthened using larger sample sizes and an accurate report of the gap between disease onset and sample collection is needed for improved data interpretation. A prospective study on the difference in days between disease onset and sample collection may prove vital towards understanding the phases of cytokine expression in response to leptospirosis infections against time. Hopefully, our data can be adopted in clinical practice as a tool for prognostic screening of patients suffering from leptospirosis infection when more definite conclusions can be drawn. This study confirmed that Th17-related IL-6, IL-17A, and IL-22 levels play pathogenic roles in the severity of leptospirosis.

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Authors' contributions SAN, MA, and ZS were involved in the conception and design of the study. WSY performed the study, analyzed data, and drafted the manuscript. AvB substantially revised the analyses and manuscript. MYY, AMS, and NMT provided clinical data and sample. LT, IK, and FA contributed intellectual content to the study. All authors read and approved the final manuscript.

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Data availability All the data supporting the conclusions of this article are included within the article.

Compliance with ethical standards

Ethical approval and consent to participate Samples were collected from study participants after they have given their written informed consent. Ethical approval for the study was obtained from the Medical Research and Ethics Committee, Ministry of Health Malaysia (NMRR (National Medical Research Register)-15-2148-27536 and NMRR-15-756-25320).

Informed consent Written informed consent was obtained from the study participants for publication of their individual details in this manuscript.

Conflict of interest The authors declare that they have no competing interests.

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