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Severity markers in severe leptospirosis: a cohort study

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Summary:

Purpose: We aimed at evaluating parameters for their value as severity markers in hospitalized leptospirosis patients.

Methods: We recruited 47 informed adult consenting patients and assessed a number of clinical, hematological, biochemical and biological variables. Patients were sorted according to severity based on the fatality or the requirement of mechanical ventilation or dialysis; the parameters studied were compared between groups on inclusion and the next day.

Results: Beside septic shock presentation or a high severity score SAPS II, increased lactate, total bilirubin, lipase, and the AST / ALT ratio or a decreased cytokines IL-10 / TNF- α ratio were all significantly associated with severity. The gene expression of the IL-1 receptor antagonist IL-1ra, the IL-1 α and the long pentraxin PTX-3 were also transcribed at higher levels in most severe cases. Patients could rapidly improve or deteriorate, highlighting the need of a new assessment the next day.

Conclusions: Our results add to the limited body of knowledge about severity markers in leptospirosis. They also suggest that patients should be re-assessed the next day before being possibly discharged from the hospital. Further studies are needed to confirm relevant and reliable prognostic parameters in leptospirosis that would be helpful for triage purpose.

Keywords:

Leptospirosis, Leptospira, Clinical signs, treatment, severity markers, Cytokines

Introduction

Leptospirosis is an infectious disease of global significance with a massive burden in tropical and subtropical regions (1, 2). Symptoms are usually a flu-like syndrome with fever, headache and myalgia and can resolve spontaneously. However, the disease can degenerate to severe forms evolving to multiple organ failures with hemorrhages, hepatic, renal and pulmonary damages related to septic shock. Case fatality rates ranging from 5% to 70% depending on clinical complications have been reported (3). Beside its endemic burden, epidemics as well as massive outbreaks have been increasingly reported over the past decades (4-8). These epidemics sometimes coincide with dengue or other epidemics (9-11). Such contexts result in limited availability of intensive care. An appropriate triage of “at high risk” patients would be helpful to optimize medical care, but is often impeded by the few prognostic factors available and / or validated for leptospirosis patients. In this work, we aimed at evaluating clinical, hematological, biochemical and other biological indicators in severe leptospirosis, using questionnaires, medical files and laboratory results in confirmed leptospirosis hospitalized patients. In a previous work, we evidenced differential gene expression patterns in inflammatory mediators between surviving and non-surviving hamsters injected with an LD₅₀, modeling the variability of leptospirosis severity (12). Therefore, besides biochemical and hematological parameters, we also studied cytokine gene expression at the transcript and protein levels.

Patients, material and methods

Patient selection and case definitions

Adult patients were recruited between March 2009 and February 2011 at Noumea Central Hospital in New Caledonia, based on clinical suspicions of leptospirosis. Three units recruited patients: Emergency (persons visiting as outpatients), Internal Medicine and Intensive Care Units. Confirmed leptospirosis cases were defined as patients with a positive PCR (13) from blood or urine or with serological evidence of leptospirosis: a four-fold increase or a seroconversion from nil to a titer of at least 800 between acute and convalescent sera using the Micro Agglutination Test. When suspicions were not confirmed, patients were excluded *a posteriori*. An additional 8 adult healthy volunteer blood donors provided blood samples for normalization of gene expression values.

Severe cases were defined as patients with either a fatal outcome or a need for mechanical ventilation or dialysis at any time of their hospital stay. Non severe patients were patients who survived with neither a need for mechanical ventilation nor dialysis.

Questionnaire and medical files

Upon recruitment, an admission form was filled by medical staff with demographical data (sex, age), date of symptom onset, clinical presentation, body temperature, current antimicrobial treatment and possible underlying condition. A form with symptoms, treatment, care (notably mechanical ventilation or dialysis) was also filled daily by medical staff for the duration of the hospital stay.

Samples and analysis

Consenting patients were sampled one 4.5 mL dry tube and one 2.5 mL PAXgene™ Blood RNA Tube venous blood daily from inclusion to Day 2 in addition to the hematological or biochemical analyses requested by the medical practitioner. Clinical scores (APACHE II and / or SAPS II) were recorded when calculated in the medical file.

Serum was collected and used for quantifying (i) the following cytokines: Tumor Necrosis Factor- α (TNF- α), Interleukin (IL-) 1 β , IL-1ra, IL-6, IL-10 and Interferon- γ (IFN- γ) and the long pentraxin 3 PTX-3 using Quantikine ELISA kits (RnDSystems); (ii) Procalcitonin (PCT) using VIDAS® (BioMérieux). PAXgene™ venous blood tubes were used for RNA extraction with the PAXgene Blood RNA kit (PreAnalytix), followed by additional DNA digestion with the TURBO DNase (Ambion); the resulting RNA was used to analyse the relative normalized gene expression (relative target gene expression normalized to the relative gene expression in healthy volunteers, using β -actin as a household reference gene) of TNF- α , IL-1 α , IL-1 β , IL-1ra, PTX-3, IL-4, IL-6, the sub-unit 40 of IL-12 (IL-12p40), IL-10 and the IFN- γ . Gene expression studies used RT-qPCR performed with the primers described in Supplementary Table 1 and 50 cycles: 95°C for 8 seconds, 60°C for 5 seconds and 72°C for 10 seconds on a LightCycler 480 using the LightCycler® 480 SYBR Green I Master kit (Roche). Sera and RNA were stored at -80°C until batch analysis in duplicates.

Statistical analysis

Data were made anonymous and captured in a database. Statistical analyses were made using SPSS 12 software (SPSS Inc., Chicago, USA). Categorical variables were expressed as percentages, while quantitative variables were described using median and percentiles. Exact Fisher's test was used for categorical variables and Kruskal Wallis one-way ANOVA for continuous variables. A p-value < 0.05 was considered significant. Two-way categorical variables were created from significant continuous

variables, allowing the calculation of a univariate odds-ratio using logistic regression. The relationships between severity and parameters were tested for data collected upon admission and for data collected the day after.

Ethics

Written informed consent was obtained from all patients. The study was endorsed by Institut Pasteur under number RBM 2008.34 after approval by Comité de Protection des Personnes Ile de France and approved by the Comité Consultatif sur le Traitement de l'Information en matière de Recherche dans le domaine de la Santé (CCTIRS) with number 09.176. The database was declared to French Commission Nationale de l'Informatique et des Libertés (CNIL).

Results and discussion

Patients

Despite a high incidence in New Caledonia, only 47 leptospirosis patients could be included in our study over a two-year period. Similar studies have been conducted with either less (14-19) or oppositely more patients (20-27). Patient demographic, clinical and selected biological data are summarized in Table 1. Because the recruitment only relied on the Central Hospital, all cases could be considered as severe leptospirosis, as milder forms would be treated in peripheral health centers. However, even in hospitalized cases of leptospirosis, there is a difference in severity and in the medical care to be implemented. We therefore categorized patients as severe or non-severe cases based on operational criteria already used in another study in Guadeloupe (21): severe cases were those who died or required dialysis and/or mechanical ventilation; oppositely non-severe cases survived with neither dialysis nor mechanical ventilation at either time of their hospital stay. Using these criteria, 22 patients were considered as “non-severe” and 25 as “severe” cases, including 4 fatalities, an 8.5% fatality rate; additionally, 4 patients initially classified as non-severe upon inclusion later deteriorated to severe. In a recent study in Brazil, severe cases were split according to the fatal outcome or recovery on one hand and to the presence of a severe pulmonary hemorrhage syndrome (SPHS) on the other hand (20). In our study, the number of fatalities or SPHS did not allow this distinction.

Patients were mostly males (31 versus 16 females) between 18.2 and 84 years old (mean 41.9, median 39.1), representative of leptospirosis in adults in New Caledonia when compared to surveillance data (8, 28). Neither the sex (Fisher $p=0.755$) nor the age (Kruskal Wallis $p=0.654$) were

related to severity. In other studies, older patients were at increased risk of severe forms or fatality (22, 24, 29-31). None of the underlying condition observed: diabetes ($p=0.611$), chronic alcoholism ($p=0.732$), obesity ($p=0.352$) or high blood pressure ($p=0.611$) proved to be significantly associated with severity. High blood pressure was shown to be a risk factor for severity in a study in Guadeloupe (21) and a risk factor for death in a study in Thailand (26). In our cohort, all 3 severe patients with an underlying high blood pressure had a fatal outcome, reinforcing these earlier findings.

The time between onset of symptoms and hospitalization was in the range 1-10 days (median 4.0) and was not associated with severity ($p=0.360$). Antimicrobial had been administered before inclusion in the study, in both severe (52.0%) and non-severe (77.3%) cases, a non-significant difference ($p=0.127$). Former studies as well as empirical knowledge consider a delayed antimicrobial treatment as a risk for severity (21, 25), a fact however not confirmed in a systematic review of clinical trials (32).

Clinical examination

Body temperature was not associated with severity ($p=0.704$, $n=42$). Most patients were febrile but 5 severe and 2 non-severe cases were hypothermic (in the range $35.4 - 36.5^{\circ}\text{C}$). Many classical symptoms of leptospirosis: icterus, hemorrhage, conjunctiva injection and a meningeal syndrome were not differentially noticed between severe and non-severe cases ($p=1.000$; $p=0.702$; $p=0.539$; $p=1.000$, respectively). In a study in France, icterus was considered a prognostic factor for severity (33). Cardiac involvement was a risk factor for mortality in a study in Guadeloupe (29) or for severity in studies in Brazil (31) and France (33); similarly, it was observed in 18.2% non-severe and 50% severe cases of our cohort ($p=0.032$). Interestingly, out of the 4 patients who deteriorated, 3 had cardiac involvement upon admission. Patients with a septic shock presentation accounted for 31.8% of the non-severe cases and 75% of severe cases ($p=0.007$), in agreement with other studies (17, 21, 26, 34, 35). Some clinical findings are the basis for our definition for severity and are therefore confounding: as a result, normal breathing, normal pulmonary auscultation and normuria were all more frequent in non-severe cases than in severe cases, mostly reflecting our severity criteria.

Hematology and hemostasis

Complete blood counts upon inclusion were not different between severe and non-severe cases ($p=0.741$ for leukocytes; $p=0.558$ for red blood cells; $p=0.388$ for platelets). In the 4 patients who later deteriorated, 3 had low platelet counts (10, 12 and 21G/L). Similarly, in the hemostasis assessment, neither the prothrombin time ($p=0.621$) nor the fibrinogen concentration ($p=0.380$) were related to severity. However, the following day, leukocyte count was higher ($p=0.037$) and red

blood cells lower ($p=0.001$) in severe cases. Similarly, the prothrombin time was higher ($p=0.035$) and the fibrinogen concentration lower ($p=0.021$) in severe patients at Day 1. This finding is in agreement with other studies which show that coagulation disorders are more frequent in severe cases, as evidenced by the prothrombin time measurement (36) and that leukocyte counts were higher in non-survivors (29). Interestingly, these severity factors were only evidenced at Day 1 in our study but were not significant upon inclusion. Similarly in a cohort study in Indonesia, Wagenaar and colleagues redefined the severity status at Day 2 (34), taking into account the possible deterioration of patients during hospitalization. During the course of hospitalization, 10 patients in our study required platelet transfusion (3 patients), packed blood cells (4 patients) or both (3 patients, including one fatality). Though this criterion is independent of our severity criteria, these 10 patients were actually recognized as severe cases.

Biochemistry

Biochemistry upon admission provided some discrimination: Lactate ($p=0.015$), LDH ($p=0.019$), bilirubin ($p=0.016$) and Aspartate aminotransferase (AST) ($p=0.049$) were significantly higher in severe cases, while Alanine aminotransferase (ALT) ($p=0.690$) or Gamma glutamyl transpeptidase (GGT) ($p=0.473$) were not different. The AST / ALT ratio already reported as a risk factor in non-survivors in a small cohort (16) was higher in severe (median 2.004) than in non-severe cases (median 1.56), a significant difference ($p = 0.008$, see Figure 1). A ratio equal or above 2 was associated with an odds ratio of 7.1 (95% CI = 1.8-28.1) for severity (see Table 2). An LDH value above 390 UI.L^{-1} was associated with an odds ratio of 5.8 (95% CI = 1.3-25.6) for severity, in agreement with a former study (25). A bilirubin concentration above $35 \mu\text{mol.L}^{-1}$ was associated with an odds ratio of 5.0 (95% CI = 1.3-20.0) for severity, in agreement with other studies (21, 24, 33). Because low lactate clearance was associated with increased mortality in severe sepsis (37), we compared the drop in lactate concentrations between inclusion and Day 1, but failed to evidence any difference between severe and non-severe cases (data not shown). In the patients deteriorating during their early hospital stay, high AST / ALT ratios (circled in figure 1), high bilirubin, high Lactate concentration, high AST or high LDH was each observed in 2 or more patients. Taken together, at least two of these parameters had values that could be considered as alerting.

Procalcitonin, a recognized marker of sepsis (38) was above the $2.5 \mu\text{g.L}^{-1}$ threshold for all cases, slightly (but not significantly, $p=0.477$) higher in severe cases (median 35 for severe, 22.6 in non-severe), both higher than levels recorded in benign leptospirosis ($1.2 \mu\text{g/L}$) in a study in France (24). The SAPS II score was only calculated for 16 patients (10 non-severe and 6 severe) but was

significantly higher in severe cases (median 56.5 and 28.5 respectively, $p=0.013$). It could prove useful to further evaluate this simplified scoring system derived from APACHE (39) in leptospirosis.

Cytokines and immune effectors

Serum concentrations of cytokines could be assessed only in a subset of patients ($n=24-30$ depending on the target), restricting the statistical power. The use of ELISA kits for measuring the concentration of several cytokines requires large volume of serum. A recent study used a multiplex bead array, an emerging technology with high analytical capacity using small volumes (20). In our study, no significant difference in serum concentration of cytokines or other immune markers was found between severe and non-severe cases, in marked contrast with other studies (14, 15, 20, 40). We notably evidenced no difference in IL-6 concentration, a cytokine widely regarded as a marker for severity in sepsis (38) and also identified as such in some (20, 23, 34), though not all (15) studies on leptospirosis. The interleukins IL-1 β , IL-2 and IL-4 were all found to be at significantly higher concentrations in severe than in mild leptospirosis in a study in Brazil (20). In our cohort a similar trend, though not significant, was noticed for IL-1 β . Oppositely, IL-2 and IL-4 were low in both severe and non-severe cases. A possible reason for our findings is that a number of patients were included after they had already been treated, including massive fluid infusion that may result in an unknown dilution of serum. Studying ratios allows neutralizing this dilution bias. Since the pioneering work of Tajiki and colleagues (14), the IL-10 / TNF α ratio was frequently evaluated in leptospirosis. This IL-10 / TNF α ratio (Figure 1) was significantly lower in severe than in non-severe cases (median 0.64, $n=14$ and median 1.15, $n=10$ respectively, $p=0.016$). An IL-10 / TNF α ratio below 1 was associated with an increased risk of severe forms (odds ratio 19.5, 95% CI = 1.8-213.9). In a very simplified view, this suggests that if the anti-inflammatory activity (as reflected by IL-10 concentration) does not counterbalance the pro-inflammatory (as reflected by TNF α), the evolution towards severe forms of leptospirosis is more likely to occur. This hypothesis is also in agreement with experiments comparing the kinetics of cytokine gene expression kinetics between susceptible and resistant animal models (12, 41). Our result is in agreement with the pioneer work of Tajiki and colleagues, but differs from a study in Greece (15). Similarly, Reis and collaborators in Brazil (20) evidenced a higher ratio in fatal than in severe non-fatal cases. This discrepancy in the results between cohort studies could be explained by the difference in the time of analysis with regard to the onset of symptoms, because cytokine expression is a very dynamic and fluctuating phenomenon, as notably evidenced in animal models (12), where kinetics are most different between susceptible and resistant models (41). Interestingly, although considered as an anti-inflammatory cytokine, IL-10 can also exert a pro-inflammatory activity enhancing the activation of natural killer (NK) cells and cytotoxic T lymphocytes

(CTLs) during endotoxemia (42). Of note, the antibiotic treatment can also trigger TNF- α over-expression, in response to *Leptospira* killing.

Gene expression techniques evidenced significant differences between severe and non-severe cases. These results are summarized in Table 3. The IL-1 receptor antagonist IL-1ra gene was expressed at higher levels in the blood of severe cases ($p=0.015$), a two-fold induction being associated with an odds ratio of 6.8 (95% CI = 1.3 – 34.6). Similarly, PTX-3 gene was more expressed in severe cases ($p=0.001$, see Figure 1), a two-fold induction corresponding to an odds ratio of 5.8 (95% CI = 1.1 – 29.9). This latter result reflects, at the transcriptional level, results formerly published by Wagenaar and colleagues about the prognostic value of the PTX-3 for both severity and fatality (34). The IL-1 α gene was also expressed at higher levels in severe cases ($p=0.025$), a two-fold increase corresponding to an odds ratio of 13.00 (95% CI = 1.4-124.3). This result corroborates our former results in a hamster model of leptospirosis, where non-survivors had a higher gene expression of this cytokine (12). TGF- β gene expression was down-regulated in all cases, whatever severity (mean relative expression ratio = 0.117), being slightly but not significantly more down-regulated in severe cases ($p=0.062$). Because mostly regarded as anti-inflammatory, this significant down-regulation might be related to our cohort, where all cases were acute leptospirosis requiring hospitalization. Lastly, no significant difference was found in the gene expression levels of TNF- α , IL-1 β , IL-6, IL-4, IL-12p40, IL-10, IFN- γ and Cox-2. The Cox-2 gene was formerly found expressed at higher levels in non-survivors in a hamster model of leptospirosis. In our cohort, a similar trend was observed, however not significant. IL-4 is known to have an anti-inflammatory activity suppressing overexpression of TNF- α , IL-1 β and IL-6 in human monocytes (43). Interestingly, although the IL-4 mRNA expression was associated with survival in patients with severe sepsis, the plasma IL-4 levels in septic patients on the day of admission to the hospital did not differ between survivors and non-survivors (44). Moreover, specific IL-4 gene deficiency in mouse had no impact on the outcome in experimental leptospirosis (45). The *Leptospira* burden was evaluated in our study using 16SrRNA quantification, as a ratio to the host housekeeping gene transcripts, as formerly done in animal models (12). Though the highest levels were observed in most severe cases, no significant difference was evidenced, mostly because of a high variability. In a former study in New Caledonia, a high bacteremia was shown to be associated with severity (25).

Conclusions:

We evidence the usefulness of using ratios for serum concentrations, as evidenced by the AST / ALT and the IL-10 / TNF- α ratios in our study, especially when massive fluid infusion have been implemented. Similarly, gene expression techniques which use ratios to normalize to reference housekeeping genes can prove useful, though not yet used in medical routine. Another concern raised in our study is a need to re-evaluate the patient the day after admission for severity, as already suggested in a former study in Indonesia (34): patients identified as non-severe at the time of admission should be kept for surveillance in a medical service before being possibly dispatched after complete re-evaluation. From inclusion to the next day, 38 patients from our cohort remained in the same severity category, only 2 improved, whereas 3 severe cases died and 4 initially regarded as non-severe deteriorated to severe. These 4 patients with rapid deterioration upon admission had at least four alerting criteria among cardiac involvement, low platelet counts, high lactate, LDH, lipase, AST, AST / ALT ratio or bilirubin (Supplementary Table 2). As an example, a woman who was not severe at the time of inclusion was kept for surveillance and required dialysis, mechanical ventilation and a transfusion of platelets the next day, finally recovering within 10 days. Not only can patients deteriorate quickly, but antimicrobial treatment can also trigger a Jarisch-Herxheimer reaction (46, 47) leading to hemodynamic support requirement. Our study provides a new independent cohort of confirmed leptospirosis patients and identifies or confirms a number of parameters that can be useful for the triage of leptospirosis patients requiring intensive care.

Limitations:

There are some limitations in our study. First, because the differential diagnosis of leptospirosis is a real clinical challenge, the parameters evidenced in our study should also be assessed for a number of other medical conditions. These conditions may vary depending on the regions but most notably include bacterial sepsis, malaria, dengue and possibly influenza.

Because patients' recruitment was only in Central hospital, all are severe leptospirosis. This recruitment strategy also restricted the number of patients informed and amenable to recruitment. Taken together, these factors have restricted the statistical power. Therefore, possible biological markers usable for prognosis may have been poorly informative in our cohort, when compared with studies that included benign leptospirosis presentations (20, 24). However, there also is a need to evaluate if a hospitalized patient can be kept under surveillance in a standard medicine unit or should be transferred to an Intensive Care Unit, especially during concurrent outbreaks leading to limited ICU availability. Thus, a better assessment of severity in hospitalized patients would also

prove useful for triage purpose. Additionally, some patients had been referred from peripheral health centers, so that not all were included at the first medical presentation. Thus, some patients were recruited after they had already been treated, including intravenous fluid infusion or antimicrobial treatment, before being recruited. Though limiting our study, this again reflects the actual medical need in hospitals, where patients may have already presented to peripheral health centers, or when leptospirosis is initially not considered as a possible etiology.

Competing interests:

The authors declare that they have no competing interests

Authors' contributions

Study conception: CG, FVP. Study design: CG, FVP, PB, FL, MM, DB. Patient recruitment and care: MM, FL, DB. Biological analyses: MESG, CM, LBO, YB, ACG. Data management: PB, MESG, CM, LBO. Analysed the data: PB, CG. Wrote the paper: CG, with contributions from all authors who read and approved the final version.

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Figure 1: AST / ALT transaminases ratio (top), IL-10 / TNF- α serum concentration ratio (center) and PTX-3 relative normalized gene expression (bottom) in severe and non-severe leptospirosis cases. Patients initially classified as non-severe but who deteriorated to severe at Day 1 are circled.

320 Table 1: Demographic, clinical and biological results for patients according to severity

	Numbers Non- severe / Severe	Normal value	Non severe			Severe			p value
			25th percentile	Median	75th percentile	25th percentile	Median	75th percentile	
Age (years)	22 / 25		32.01	40.55	50.91	24.06	38.97	57.86	0.654
Days since onset	21 / 22		2.00	4.00	4.50	3.00	4.00	5.00	0.360
SAPS II score	10 / 6		14.75	28.50	34.00	42.75	56.50	98.00	0.013
Lactate (mmol/L)	10 / 20	0.3-1.3	1.37	1.76	2.23	2.00	2.80	3.87	0.015
Total Bilirubin (μmol/L)	20 / 24	3.4-17	25.25	40.00	172.75	56.75	146.50	328.25	0.016
AST (GOT) (IU/L)	21 / 24	5-34	47.50	76.00	167.00	87.50	127.50	213.50	0.049
ALT (GPT) (IU/L)	21 / 24	<55	40.00	64.00	92.50	49.00	62.00	92.75	0.690
AST / ALT ratio	21 / 24		0.95	1.565	1.86	1.58	2.004	2.40	0.008
Lipase (IU/L)	15 / 15	5.6-	13.00	55.00	112.00	24.00	154.00	436.00	0.050

		51.3							
LDH (IU/L)	19 / 23	100-350	188.00	277.00	368.00	276.00	395.00	515.00	0.019
Procalcitonin (µg/L)	14 / 15	<0.01	5.55	22.58	55.11	15.99	35.00	42.29	0.477
TNF-α (pg/mL)	13 / 14	<15.6*	9.70	81.60	153.60	29.58	112.55	197.70	0.325
IL-1β (pg/mL)	13 / 12	<3.90*	0.00	0.00	1.51	0.00	3.70	10.10	0.101
IL-1ra (pg/mL)	12 / 14	360 +/- 214*	2547.25	2706.10	3633.73	2555.25	2646.50	2710.85	0.347
PTX-3 (ng/mL)	13 / 14 19 / 15	0.65*	37.10 22.63	60.81 40.25	64.76 54.30	39.83 42.95	49.20 48.50	62.91 59.15	0.458 <i>(0.023 at Day 1)</i>
IL-6 (pg/mL)	12 / 13	1.77 (<10)*	7.43	17.31	22.90	1.80	10.34	17.60	0.087
IL-10 (pg/mL)	13 / 14	<3.47*	40.87	69.25	169.70	13.42	24.65	119.40	0.128
IL-10 / TNF-α ratio	10 / 14		0.62	1.15	3.52	0.19	0.64	0.88	0.016

321 * no normal value is clinically validated. Data indicated are values in sera of healthy volunteers according to the manufacturer.

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323 Table 2: Categorical variables and corresponding univariate odds ratio where significant.

	Numbers Non-severe / Severe	Proportion in Non-Severe (%)	Proportion in Severe (%)	p-value (Fisher exact or * χ^2)	univariate Odds ratio	95% CI
Septic shock presentation	22 / 24	31.8	75.0	0.007	6.4	1.8 – 23.3
AST / ALT ≥ 2	21 / 24	14.3	54.2	0.006	7.1	1.8 – 28.1
Total Bilirubin ≥ 35	20 / 24	50.0	83.3	0.025	5.0	1.3 – 20.0
LDH ≥ 390	19 / 23	15.8	52.2	0.023	5.8	1.3 – 25.6
serum IL-10 / TNF- α ratio <1	10 / 14	40.0	92.9	0.009	19.5	1.8 – 213.9
IL-1 α gene expression ≥ 2	14 / 16	7.1	50.0	0.017	13.0	1.4 – 124.3
PTX-3 gene expression ≥ 2	14 / 16	42.9	81.3	0.029*	5.8	1.1 – 29.9
IL-1ra gene expression ≥ 2	13 / 16	30.8	75.0	0.027	6.8	1.3 – 34.6

324

325

326 Table 3: Gene expression of cytokines and other immune markers according to severity

	Numbers Non-severe / Severe	Non severe			Severe			p value
		25th percentile	Median	75th percentile	25th percentile	Median	75th percentile	
TNF- α	14 / 16	0.51	1.22	2.32	0.56	1.84	3.44	0.313
IL-1 β	13 / 16	0.42	0.92	1.62	0.36	0.78	1.64	0.846
IL-1ra	13 / 16	0.58	1.29	3.05	1.66	4.10	6.98	0.015
PTX-3	14 / 16	0.38	1.62	3.38	3.38	5.40	12.43	0.001
IL-6	14 / 16	0.01	0.09	0.19	0.08	0.26	0.42	0.110
IL-1α	14 / 16	0.16	0.80	1.52	0.74	1.96	4.19	0.025
IL-12p40	14 / 16	0.22	0.33	1.03	0.22	0.55	1.41	0.179
IL-10	13 / 16	1.63	3.99	22.70	5.19	10.89	24.80	0.156
IFN- γ	13 / 14	0.02	0.10	0.59	0.12	0.37	1.21	0.105
TGF- β	13 / 16	0.06	0.11	0.12	0.05	0.06	0.07	0.062
Cox-2	13 / 16	0.40	1.06	1.26	0.43	1.43	2.12	0.170
<i>Leptospira</i> 16S to human household	12 / 14	0.01	3.95	802.25	0.00	54.77	3867.50	0.494

327 Results are relative normalized expression ratio as detailed in the text, where normal values are 1.

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