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Neutrophil counts in leptospirosis patients infected with different serovars

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Abstract. In leptospirosis patients, haematological abnormalities have been reported. The aim of this study was to determine if neutrophil counts were different between patients known to be infected with a range of leptospiral serovars. The study retrospectively compared the neutrophil counts from the first blood samples taken from 210 leptospirosis patients at first presentation to a Queensland Health hospital. Significant differences ($p < 0.001$) were observed in neutrophil counts across the 11 different infecting serovars. These findings suggest that neutrophil counts may be useful in the development of an algorithm determining the infecting serovar in suspected leptospirosis patients. Further studies are required to delineate host cytokine responses which may suggest the underlying aetiology of the observed differences in neutrophil counts. Such studies would also provide valuable therapeutic insights into treating the disease.

INTRODUCTION

Leptospires, the biological agents responsible for leptospirosis are gram negative, catalase and oxidase positive spirochetes approximately 6-20 µm in length (Levett, 2001). A leptospiral infection is usually acquired through either direct or indirect contact with infected water, soil, vegetation or urine from an infected animal. Infection occurs through cuts and abrasions in the integument or exposed mucous membranes (Levett, 2001). Leptospiral infections in humans can vary from being mild, through to catastrophic as in Weil's disease where, in extreme cases, the onset of renal and hepatic failure can occur (Dall'Antonia *et al.*, 2008). Weil's disease (also referred to as severe icteric leptospirosis with renal failure) has a mortality rate of 5-50% (Alston & Broom, 1937; Levett, 2001).

The diagnosis of leptospirosis is arduous due to the observation that none of the presenting features (headeache, fever, myalgia, arthralgia, nausea, vomiting, haemoptosis, photophobia) are specific for the disease. The biphasic nature of the disease may also hinder a rapid diagnosis. During the acute phase of the disease, leptospires need to be targeted with methods such as PCR and blood culture in Ellinghausen McCollough Johnson Harris (EMJH) media. During the immune phase serum antibodies need to be targeted with the Microscopic Agglutination Test (MAT). The use of a Leptospiral IgM ELISA is beneficial in the immune phase and the acute phase if there is any uncertainty which stage of the disease the patient may be presenting in (Craig, 2011).

A number of reviews and studies have been undertaken in recent years to better

understand the value of laboratory findings for the diagnosis and management of leptospirosis (Esen *et al*., 2004). Lymphopenia during the acute phase of leptospiral infections appears common across the majority of pathogenic serovars screened for in Australia with *L. borgpetersenii* serovar Arborea, *L. borgpetersenii* serovar Hardjo and *L. interrogans* serovar Copenhageni being the possible exceptions (Craig *et al*., 2009). Haemoglobin and red cell count results from the first blood samples taken from patients in the acute phase of leptospirosis show significant differences in haemoglobin and red cell counts in those infected with *Leptospira interrogans* serovars Szwajizak and Canicola when compared with most of the other serovars (Craig *et al*., 2013).

While there is now a slow accumulation of published data in relation to lymphocyte, haemoglobin and red cell counts between different infecting leptospiral serovars, data on other haematological markers such as neutrophil counts in patients with different infecting serovars appears to elude the research literature. The aim of the study was to determine if neutrophil counts were different between patients infected with different leptospiral serovars at first presentation.

MATERIALS AND METHODS

The study protocol was approved by the Human Ethics Committee from Queensland Health Forensic and Scientific Service (Approval Number 08-001/12) and the Human Ethics Research Committee from the University of the Sunshine Coast (Approval Number A/08/155).

A total of 210 leptospirosis patients, between 18-75 years of age were identified and investigated retrospectively over a ten year period (1999–2009) using the patient database at the WHO/FAO/OIE Collaborating Center for Reference and Research on Leptospirosis, Brisbane. Leptospirosis was confirmed through the isolation of leptospires from blood cultures in EMJH media, detection by real time PCR, or serology with a microscopic agglutination test (MAT) showing a four fold (or greater) rise in titre on follow up from the initial presentation. At the time of presentation all patients were MAT non reactive indicating acute phase of the disease. Patients presenting with severe leptospirosis for example, significant respiratory distress, indicated by diffuse alveolar haemorrhage and/or acute liver or renal failure requiring admission to an intensive care unit or high dependency unit were excluded from the study.

Common diseases causing pyrexia in Australia such as dengue fever, Ross River fever, infections by Barmah Forest Virus and rickettsial species were excluded through serology while infections with pathogenic *Staphylococcus* spp., *Meningococcus* spp., *Pseudomonas* spp., *Haemophilus* spp., and other anaerobes were excluded by negative blood cultures.

Pathology results reported were those from the first sample collected at the initial presentation at a Queensland Health Hospital. Neutrophil counts between the different serovar infected groups were compared firstly using a between groups analysis of variance (ANOVA). Derived F statistics <0.05 were followed up with *post hoc t*–Tests using the *t*-Tests statistical function in Microsoft Excel. To prevent Type 1 statistical errors due to the number of pairwise comparisons, neutrophil counts between the groups were considered significant for P values < 0.01.

RESULTS

Neutrophil count results from patients infected with different serovars are presented in Table 1. Significant differences were observed (Table 1) in neutrophil counts (F = 7.1; p= <0.001) across the serovars.

The comparisons of the mean neutrophil counts between the different infected serovar groups revealed that the higher mean neutrophil count observed in the serovar Robinsoni infected group was significantly different to most of the other serovar infected groups except for groups infected with serovars Australis, Kremastos, Szwajizak and Zanoni (Table 2). The higher mean neutrophil count observed in the serovar Zanoni infected

group was also significantly different to most of the other serovar infected groups except for groups infected with serovars Australis and Robinsoni (Table 2). Similarly, the higher mean neutrophil count observed in the serovar Australis infected group was significantly different to most of the other serovar infected groups except for groups infected with serovars Robinsoni, Szwajizak and Zanoni (Table 2).

DISCUSSION

In regards to Leptospirosis there is significant published data in relation to lymphocyte, erythrocyte and haemoglobin counts between different infecting serovars (Craig *et al*., 2009; Craig *et al*., 2013). However, there is a paucity of data about the variation of other haematological markers between different infecting serovars in the Leptospirosis literature. The aim of this study was to compare and identify neutrophil counts between patients infected with different leptospiral serovars at first presentation.

Neutrophils play an important role in the phagocytic uptake and intracellular destruction of microbial pathogens (Nathan, 2006). In septic patients the binding of platelets to neutrophils may facilitate the release of chromatin and azurophilic and gelatinase granules to form neutrophil extracellular traps in the microvasculature for the trapping and destruction of organisms (Brinkman *et al*., 2004; Ma & Kubes, 2008). The actual mechanism underpinning the observed increase in neutrophil counts in the serovars Australis, Robinsoni and Zanoni infected groups remains to be elucidated. One possibility is neutrophil exposure to granulocyte colony stimulating factor (G-CSF). G-CSF promotes proliferation of neutrophil precursors and the release of neutrophils into the circulation from the bone marrow (Kaushansky, 2006). It has been reported that inflammatory cytokines such as tumour necrosis factor α (TNF-α), interleukin 1 (IL-1) and interleukin 6 (IL-6) produced by activated monocytes stimulate G-CSF production (Kaushansky, 2006). Recently, Wang *et al.* (2012) reported that leptospiral haemolysins Sph1, Sph2, Sph3, HIpA and YIyA from *L. interrogans* strain Lai induced a strong production of TNF-α, IL-1β and IL-6 in human and mouse macrophages via Toll-Like receptors activating the JNK and NF-κB pathways.

There is currently a major, collaborative international effort to sequence numerous leptospiral genomes from a number of geographical locations. Once this data is complete, haemolysin gene and putative protein comparisons between different serovars and strains should provide insights into the differences observed in this study.

Table 1. Neutrophil count as a function of infecting serovar

Serovar	Neutrophils (x 10^9 / L)*		
	n	Mean	S.E.
L. borgpetersenii serovar Arborea	14	5.43	2.62
L. interrogans serovar Australis	47	8.48	3.16
L. interrogans serovar Canicola	7	5.39	1.57
L. weilii serovar Celledoni	6	4.74	1.49
L. borgpetersenii serovar Hardjo	15	5.17	2.05
L. interrogans serovar Kremastos	15	6.11	2.49
L. interrogans serovar Robinsoni	18	9.19	4.40
L. interrogans serovar Szwajizak	8	6.77	1.94
L. borgpetersenii serovar Tarassovi	10	4.84	2.26
L. weilii serovar Topaz	9	4.50	1.72
L. interrogans serovar Zanoni	67	9.36	3.77
F =		7.1	
P =		<0.001	

* Normal reference range = 2-8 x 10^9 / L

Table 2. *P* values for mean pairwise serovar neutrophil comparisons

Serovar	Arborea	Australis	Canicola	Celledoni	Hardjo	Kremastos	Robinsoni	Szwajizak	Topaz	Tarassovi	Zanoni
Arborea	–	**0.001**	0.97	0.46	0.77	0.47	**0.006**	0.19	0.32	0.57	**<0.001**
Australis	–	–	**0.001**	**<0.001**	**<0.001**	**0.006**	0.54	0.06	**<0.001**	**<0.001**	0.19
Canicola	–	–	–	0.45	0.78	0.42	**0.004**	0.15	0.29	0.56	**<0.001**
Celledoni	–	–	–	–	0.06	0.14	**0.001**	0.05	0.78	0.091	**<0.001**
Hardjo	–	–	–	–	–	0.27	**0.002**	0.08	0.40	0.71	**<0.001**
Kremastos	–	–	–	–	–	–	0.02	0.49	0.07	0.21	**<0.001**
Robinsoni	–	–	–	–	–	–	–	0.63	**<0.001**	**0.002**	0.88
Szwajizak	–	–	–	–	–	–	–	–	0.02	0.07	**0.007**
Topaz	–	–	–	–	–	–	–	–	–	0.71	**<0.001**
Tarassovi	–	–	–	–	–	–	–	–	–	–	**<0.001**
Zanoni	–	–	–	–	–	–	–	–	–	–	–

Alternatively, the mechanism responsible for the increase may be the release of interleukin 17 from lymphocytes to stromal cells in the bone marrow thus stimulating G-CSF release (Nathan, 2006). This alternative however maybe less tenable given that lymphopenia is a common finding in the acute phase of leptospirosis (Craig *et al*., 2009).

Haematological markers are beginning to reveal differences between common infecting leptospiral serovars. From the data above, on average, high neutrophil counts appear in those infected with serovars Robinsoni, Zanoni and Australis. From the literature, erythrocyte and haemoglobin counts appear higher in those infected with serovar Szwajizak and lower in those infected with serovar Canicola (Craig *et al*., 2013). Further, lymphopenia appears absent from those infected with serovars Arborea, Hardjo, and Copenhageni (Craig *et al*., 2009). With the study of additional haematological markers it is hoped that an algorithm will be possible to differentiate infecting serovars at the time the patient first presents. Such and algorithm will be beneficial for empirical treatments and patient management particularly in areas with limited medical resources.

In conclusion, this study compared neutrophil counts in patients infected with different leptospiral serovars at first presentation. Differences between groups of patients infected with different serovars were identified in relation to neutrophil counts. While leptospiral haemolysins may induce cytokines to release G-CSF, further studies are required to delineate host cytokine responses which may suggest the underlying aetiology of the observed differences in neutrophil counts. Such studies would also provide valuable therapeutic insights into treating the disease.

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