

CASE REPORT

First observation of neutrophil extracellular traps in human leptospirosis

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ABSTRACT

Leptospirosis is the most important global zoonosis and is caused by pathogenic spirochetes of the genus *Leptospira*. Human leptospirosis ranges in severity from a mild, self-limited febrile illness to a fulminant life-threatening one but their pathogenesis is still unclear. The extracellular release of the nuclear DNA of neutrophils, called NETs, upon activation by microbes is a pathogen-killing mechanism of neutrophils described in 2004 although its presence in human pathology have been observed only very recently. We report a case of fatal fulminant leptospirosis with associated severe pulmonary involvement and shown for the first time, evidence of the presence of NETs in the lung tissue.

Key Words: Neutrophils, Elastase, Pulmonary hemorrhage, *Leptospira*, Pathogenesis

1. INTRODUCTION

Leptospirosis is a global zoonosis caused by pathogenic spirochetes of the genus *Leptospira*.^[1] Recently, there have been estimated more than 1,000,000 cases and near 60,000 deaths due to leptospirosis worldwide per year.^[2]

Human leptospirosis ranges from a mild to a fulminant mortal one. Several organ systems may be involved. As a result, the signs and symptoms of leptospirosis are highly varied and frequently mistaken for other causes of acute febrile syndrome.^[3]

Usually leptospirosis cases are mild and resolve spontaneously (>90%). A low percentage of cases (<10%) progress to septicemia with multisystem organ failure.^[3] In these cases, hemorrhagic alterations are frequently observed. Severe pulmonary hemorrhagic syndrome has a fatality rate of

>50%.^[3]

The extracellular release of the nuclear DNA of neutrophils upon activation by microbes is a relatively novel pathogen-killing mechanism of neutrophils.^[4] These DNA structures, named neutrophil extracellular traps (NETs), are composed of chromatin, associated with several proteins with antimicrobial properties such as citrullinated histones, elastase and myeloperoxidase among others, and serve as a physical barrier that prevents the further spreading of pathogens.^[5]

Although it was originally proposed that NETs are formed exclusively in tissues at sites of bacterial or fungal infection, NETs have also been found within blood vessels where they ensnare circulating bacteria during experimental sepsis.^[6] The presence of NETs in human leptospirosis is unknown.

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2. CASE PRESENTATION

A 70-year-old man presented with jaundice, fever (39°C), generalized fatigue, conjunctival suffusion, petechial rash and looked severely ill. Upon admission, he was transferred to the intensive care unit (ICU). Respiratory sounds were low in both lung fields. Chest X-rays showed a scattered nodular infiltrate. Arterial blood gases were pH 7.42, pCO₂ 27.5, pO₂ 37.5 mmHg, and oxygen saturation 72.1%. The patient presents also the following laboratory data: white blood cells (μl) 14,100; platelet count (μl) 22,000; total bilirubin (mg/dl) 18, and direct bilirubin (mg/dl) 14. The patient was mechanically ventilated with 100% oxygen. However, his general condition become worse and died twelve hours after admission. A relative of the patient described the presence of a rat at his apartment. His wife was admitted one day later with typical Weil's syndrome. She was treated and presented full recovery. A serological diagnosis confirmed *Leptospira interrogans* serovar Icterohaemorrhagiae in her case.

2.1 Histology and bacterial antigen detection

At necropsy, marked pulmonary hemorrhage was seen. Search for leptospiral antigen in lung tissues using antibodies against *Leptospira interrogans* was intended using an immunohistochemical procedure that have been described before.^[7]

2.2 NETs detection

As regarding NETs detection, a few modifications for a better visualization in paraffin embedded tissues using a immunofluorescence (IF) technique were recently published^[5] and applied in this case. Rabbit polyclonal against human neutrophil elastase (NE) (Calbiochem-Merk Millipore - Darmstadt, Germany), or citrulinated histone 3 (H3cit) (Santa Cruz, Ca, USA), followed by anti species antibody labeled with different fluorochromes were used. Nuclei were counterstained

with DAPI (Invitrogen, Argentina). In all samples, they were observed under a Nikon E200 photomicroscope.

2.3 Results

The histologic sections of the lung showed extensive alveolar hemorrhage (see Figure 1A), with early prominence of alveolar lining, mild interstitial edema, and presence of some histiocytes-macrophages and neutrophils in the alveoli and interstitium (see Figure 1B).

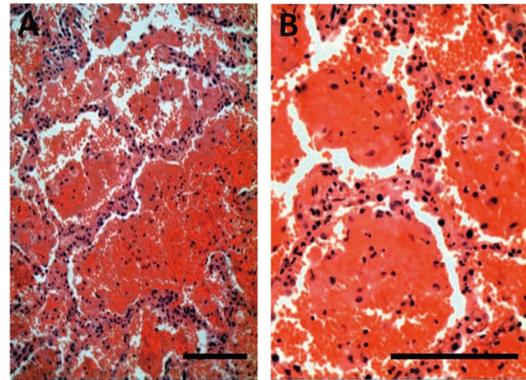


Figure 1. Pulmonary pathology. (A) Lung tissue shows widespread hemorrhage and infiltrates involving alveoli and septa. (B) Neutrophils and macrophages are present in the alveoli and interstitium. Hematoxylin and eosin staining. Scale bar indicates 50 μm.

Although in absence of the primary antibody the staining was negative (see Figure 2A), positive antigen reactions of *Leptospira* sp., inside of macrophagic cells were seen when the antibody against *Leptospira* sp. was used (see Figure 2B). When anti NE was used, an important number of disseminated infiltrating neutrophils were identified as part of the cell exudate (see Figure 2C).

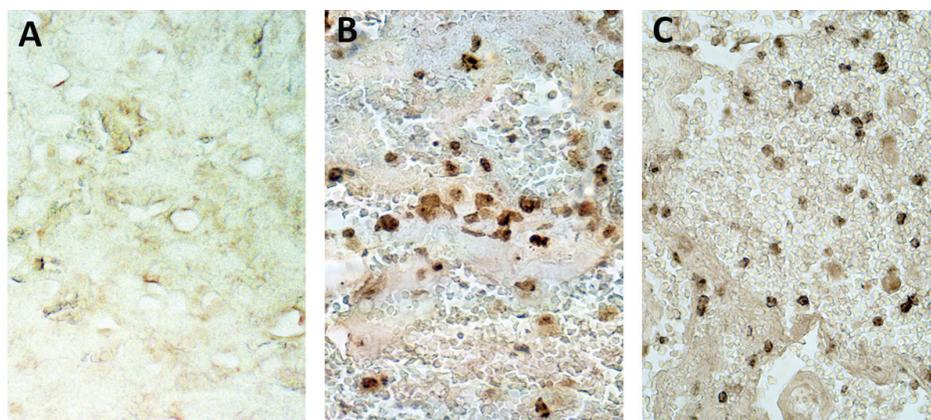


Figure 2. *Leptospira* and neutrophil detection. (A) Negative staining in absence of primary antibody. (B) Strong staining for *Leptospira* antigen in infiltrated cells. (C) Infiltrating neutrophils in interalveolar septum and alveoli detected by neutrophil elastase staining. Immunohistochemistry. Scale bar indicates 50 μm.

In order to detect NETs, a dual staining of NE and H3cit was intended. The IF technique allowed to detect colocaliza-

tion of both antigens in the same structure (see Figure 3A-B, Pearson correlation coefficient = +0.4572).

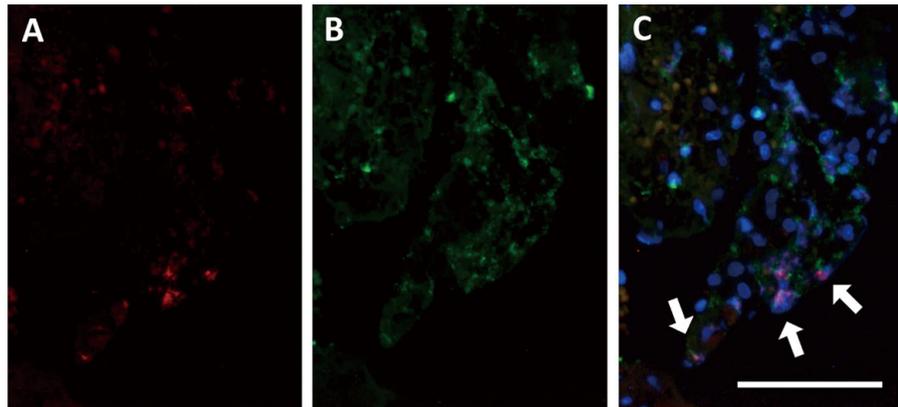


Figure 3. NETs in lung tissue of human leptospirosis. (A) a neutrophil-rich area stained for citrullinated H3 (red); and (B) neutrophil elastase (green) in relaxed chromatin of netting neutrophils and NETs. (C) Merged images showing colocalization of granular and nuclear components (yellow) and DNA (blue) staining. Arrows indicating the NETs-positive area. Immunofluorescence microscopy. Scale bar indicates 50 μm .

3. DISCUSSION

The histology, as revealed by the H&E staining, was compatible with the clinical history of the patient. The applied immunohistochemistry procedure was extremely useful not only in supporting the diagnoses but also in demonstrating leptospiral antigen, mostly as phagocytosed material present in the cytoplasm of macrophagic cells in the affected areas. This distribution of the bacterial antigen was very similar to that found in a reported case study.^[9] The abundance of neutrophils in the cell exudate as showed by the NE immunostaining, is not surprising since leptospirosis in humans is frequently associated with neutrophilia.^[3] In the past, the presence of NETs in tissues, particularly in paraffin embedded samples, was hardly identified, and staining for just one component, e.g., DNA, was considered not sufficient.^[5]

Our observation of colocalization of granular proteins in the nucleus and the citrullination of histones provide unique features for neutrophils undergoing NETosis that can be exploited for identification of these cells in tissue sections as it has been recently described.^[5] In this regard, we have recently described that *Leptospira interrogans* may induce NETs in human neutrophils and that pathogenic *Leptospira*

has the ability to escape from NETs.^[8] However, the presence of NETs in human leptospirosis was not described previously.

Although aspects as initial inoculum size and difference in the host defenses are considered critical, the pathogenesis of leptospirosis is still uncovered. It is still not known the reason why some patients course a fulminant pulmonary hemorrhagic form and others do not, as was the case of the wife of the deceased patient.

In the best of our knowledge, this is the first demonstration of NETs in human leptospirosis. Due to their pathogenic potential, identification of NETs in tissue samples from patients with leptospirosis is important and could be of diagnostic value in the future.

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CONFLICTS OF INTEREST DISCLOSURE

All authors declared no competing interests.

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