The emergence of severe pulmonary hemorrhagic leptospirosis: questions to consider

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Since the 1980s, the incidence of severe pulmonary hemorrhage caused by *Leptospira* spp. infection has increased. The mild, non-specific symptoms or the more classical form of severe disease with hepatorenal manifestations, Weil's syndrome, predominate worldwide. However, several regions of the world have seen increases in numbers of patients with pulmonary hemorrhage attributed to leptospirosis. The reasons behind the emergence of this syndrome, which carries a high mortality rate, are not known. Several avenues for future research may shed light on the mechanisms involved in development of pulmonary hemorrhage, and inform targeted therapeutics to improve outcomes. Possibilities to consider include: (1) emergence of new bacterial strains, (2) acquisition of virulence traits by strains in the endemic regions, (3) changes in underlying health of the affected human populations, and (4) increased recognition of the syndrome and better record keeping by the medical and veterinary communities. Determining the causes of emerging clinical manifestations presents challenges and opportunities for potentially life-saving research into the pathogenesis of a number of infectious diseases, including leptospirosis.

Keywords: leptospirosis, *Leptospira*, pulmonary hemorrhage

INTRODUCTION

Leptospirosis is the clinical manifestation of zoonotic infection by spirochetes of the genus *Leptospira* (reviewed in Adler and de la Pena Moctezuma, 2009; see also http://www.who.int/zoonoses/diseases/lerg/en/index.html). While several *Leptospira* species can cause infection and disease in humans and animals, the severity of disease is multifactorial. In humans, leptospirosis may range from a very mild and self-limited illness to severe multisystem illness that includes high fever, renal failure, jaundice, and aseptic meningitis as well as a plethora of other signs and symptoms. Weil's syndrome, a severe leptospirosis manifestation, is characterized by renal failure, jaundice, and splenomegaly. Traditionally, those most at risk reside in tropical, developing countries, although the disease is geographically widespread. Traditionally, certain occupations, including farming/ranching, veterinary medicine, abattoir, or sewer work, place individuals at increased risk of leptospirosis. Currently, however, leptospirosis is considered by many to be a re-emerging infectious disease due primarily to exposure to rats and their urine in urban slum settings, and exposure through recreational activities and flooding to water contaminated by animal urine.

Severe pulmonary hemorrhagic leptospirosis (SPHL; Dolhnikoff et al., 2007) as a presentation of leptospirosis has also been termed severe pulmonary hemorrhagic syndrome (SPHS; Ko et al., 1999; Gouveia et al., 2008), severe pulmonary form of leptospirosis (SPFL; Silva et al., 2002; Spichler et al., 2008; Marchiori et al., 2011), and leptospiral pulmonary hemorrhage syndrome (LPHS; Croda et al., 2010). SPHL has been recognized as an emerging clinical manifestation over the last two decades. While hemorrhagic and pulmonary manifestations of leptospirosis have long been recognized (reviewed in Edwards and Domm, 1960; da Rocha Medeiros et al., 2010), SPHL carries a high mortality rate, and does not always coincide with the classic manifestations of severe leptospirosis (Weil's syndrome). On autopsy, patients with SPHL generally have hemorrhagic and/or necrotic lesions in numerous other sites (Chen et al., 2007; Spichler et al., 2007), suggesting that SPHL might be simply the most easily, externally discernable sign of generalized hemorrhage.

The manifestations of SPHL reported by several different groups include dyspnea, crackles, and sometimes massive hemoptysis, radiographic findings such as alveolar infiltrates not associated with any particular lobes of the lung, and rapid deterioration of the patient's condition such that mechanical ventilation is required (see Silva et al., 2002; Gouveia et al., 2008; Spichler et al., 2008; Marchiori et al., 2011). Even with intensive supportive care and antibiotic therapy, once SPHL has developed, outcome is poor. The reasons behind the emergence of SPHL are not yet known, but are important to understand in order to decrease morbidity and mortality in leptospirosis patients.

POSSIBLE MECHANISMS DRIVING THE EMERGENCE OF SPHL

Possibilities to consider regarding the emergence of SPHL are manifold. Certain *Leptospira* species and serovars are endemic to particular regions, although introduction of a new strain can cause increased rates of leptospirosis in endemic regions (Thaipadungpanit et al., 2007). Although this cannot be directly tested, many in the field believe that certain *Leptospira* species and serovars (*Leptospira interrogans* serovars Copenhageni, Icterohaemorrhagiae, Lai) have tendencies to cause greater severities of disease in humans (Faine et al., 1999; Adler and de la Pena Moctezuma, 2009). However, the geographic diversity of SPHL does not support

the phenomenon being purely regional or attributable to any specific leptospirosis agent. A number of different *L. interrogans* serovars have been associated with pulmonary hemorrhage (Trevejo et al., 1998; Seijo et al., 2002; Vijayachari et al., 2008), although it has been contested that overall there is poor correlation between particular serovars and severity of leptospirosis (Vinetz, 2001). Genome sequences have been published for several leptospirosis agents, including *L. interrogans* Copenhageni and Lai (Ren et al., 2003; Nascimento et al., 2004), as well as two strains each of *Leptospira borgpetersenii* serovar Hardjo-bovis (Bulach et al., 2006) and the saprophyte *Leptospira biflexa* serovar Patoc (Picardeau et al., 2008). It is possible that, as additional *L. interrogans* genome sequences are determined, polymorphisms will be correlated with SPHL. In an ambitious undertaking aimed at better understanding the pathogens of the genus *Leptospira*, sequencing of approximately 200 different isolates is underway (http://gsc.jcvi.org/projects/gsc/Leptospira/index.shtml).

Comparison of two *L. borgpetersenii* serovar Hardjo genome sequences reveled interesting avenues to purse in future investigations into the biology and pathogenesis of the genus *Leptospira* (Bulach et al., 2006). Both strains are able to cause infection in some host species, but hamster susceptibility to lethal infection by the two strains differs. Both genomes are smaller than those of *L. interrogans* serovars Lai and Copenhageni. The authors found that most striking differences lie in genes that enable the bacteria to survive in different environments, e.g., those whose products are involved in biosynthesis and transport of nutrients, and in regulation of gene expression. This affected the ability of *L. borgpetersenii* Hardjo, as compared to *L. interrogans*, to survive in water. The findings of this work are also consistent with the epidemiology, in that *L. borgpetersenii* appears to require direct transmission to new hosts.

As genome sequencing becomes more rapid and facile for minimal cost, the focus will need to evolve to identifying functions for predicted hypothetical proteins and those known to be produced, but for which no function is known. It is possible that, within any geographic region, there are clones belonging to a single species and serovar that have emerged with heightened virulence due to altered genome sequences. The large sequencing project will be particularly informative inn this regard. This would be one example of independent convergent evolution, although it is difficult to discern how causing SPHS would benefit organisms that rely on maintenance of infection in reservoir hosts. It is possible, however, that such mutations may have minimal or even beneficial effects on the organisms in non-human environments.

To definitively determine the importance of any candidate virulence attribute, the genetic basis of *Leptospira* species virulence will need to be determined at the level of gene function and regulation. Recent developments in the genetic manipulation of *Leptospira* species (Bauby et al., 2003; Bourhy et al., 2005; Louvel and Picardeau, 2007; Croda et al., 2008; Ko et al., 2009; Murray et al., 2009; Aviat et al., 2010; Poggi et al., 2010) will facilitate identification of genes whose products are essential for, or contribute to, pathogenesis of leptospirosis. In a groundbreaking demonstration of the power of this approach, the gene encoding an OmpA-like outer membrane protein, *loa22*, was demonstrated to contribute to the ability of *L. interrogans* serovar Lai to cause

disease in hamsters and guinea pigs (Ristow et al., 2007). Recombinant Loa22 is reported to have a number of effects on rat kidney cells in culture (Zhang et al., 2009), but the biological significance is difficult to discern at this point. More recently, a nudix hydrolase was shown to contribute to virulence of *L. interrogans* serovar Lai in hamsters (Luo et al., 2011). Interesting additional candidates to pursue will include the putative sphingomyelinases annotated in the genome, the pore-forming toxin SphH (Lee et al., 2000, 2002), and the adhesins that mediate attachment to host molecules (Merien et al., 2000; Palaniappan et al., 2002; Matsunaga et al., 2003; Barbosa et al., 2006; Verma et al., 2006; Choy et al., 2007; Stevenson et al., 2007; Atzingen et al., 2008).

It is possible that some of the disease manifestations will eventually be linked to particular virulence attributes, and to proteins responsible for direct damage or for expression of particular genes or regulons. A possible route for investigation of the effects of possible leptospiral disruption of cells or tissues comes from work published by Croda et al. (2010), which demonstrated that, in lungs of patients who died of severe leptospirosis, pulmonary hemorrhage correlated with deposits of immunoglobulins and complement component C3. Deposits of immunoglobulins and C3 were not seen in patients with non-leptospirosis associated pulmonary hemorrhage. This phenomenon was first noted in the guinea pig model of leptospirosis (Nally et al., 2004), and together the findings suggest a significant role for the immune response in the development of SPHL. However, the early presentation of SPHL might suggest that direct damage by bacterial factors may play a role (Silva et al., 2002; Vijayachari et al., 2008). *In vitro*, *L. interrogans* serovars Canicola and Copenhageni were demonstrated to disrupt endothelial layers in culture (Martinez et al., 2010). The mechanisms of damage underlying these findings will be interesting to investigate.

In addition to polymorphisms in gene sequences, strain-to-strain variations may also be due to mutations that affect regulation of virulence gene expression and protein production, or due to acquisition of additional genomic sequences from other organisms. Since leptospirosis is a zoonotic infection that is often water-borne, there are abundant opportunities for leptospires to interact with other microorganisms and perhaps to exchange genetic information. Given the widespread geographic distribution of SPHL, however, it is does not appear likely that acquisition of any particular new genetic element is the underlying reason for the emergence of this leptospirosis manifestation, unless a donor species can be demonstrated to survive in similar environments world-wide. It has been demonstrated, however, that *E. coli* can transfer a derivative of a broad host-range conjugal plasmid to *L. interrogans* and *L. biflexa* in the laboratory (Picardeau, 2008), so the possibility does exist.

Further understanding of the host side of the *Leptospira*–human interaction in the emergence of SPHL will also merit further investigation. The patients affected by SPHL have not been consistently found to have significant gender bias apart from the historic overall male bias in leptospirosis that is in part due to the occupations and activities that increase risk for leptospirosis. One notable exception to this trend is that in Salvador, Brazil, Gouveia et al. (2008) reported that, although male patients predominated in the study population, female patients had a higher

risk of developing SPHL. SPHL is seen in previously healthy adults and children, suggesting that age is not a significant factor in SPHL development, but may affect the fatality rate (Marotto et al., 1997). Reports from Brazil, France, India, Israel, Korea, Nicaragua, Peru, Taiwan, Thailand, and Vietnam document SPHL as a manifestation of leptospirosis (Park et al., 1989; Zaki and Shieh, 1996; Marotto et al., 1997; Borer et al., 1999; Chen et al., 2007; Spichler et al., 2007, 2008; Gouveia et al., 2008; Vijayachari et al., 2008; Clavel et al., 2010), although the rates have varied in the different studies and locations. One study has identified a possible genetic link to susceptibility to leptospirosis (*not* SPHL, specifically; Lingappa et al., 2004). In this set of patients in a large US outbreak among triathletes, an association was found between HLA-DQ6 plus ingestion of lake water and leptospirosis. This finding has not yet been further tested in other, larger outbreaks, such as that in Manila, Philippines in the fall of 2010. The HLA-DQ6–water ingestion–leptospirosis association may also be a function of the particular species and serovar that caused the outbreak. It is therefore not yet clear whether genetic traits in the human population in any particular location play significant roles in susceptibility to SPHL or any other manifestation of leptospirosis. Given the complexity of leptospirosis, and the lack of co-incidence between SPHL and the more classical severe leptospirosis signs and symptoms (Weil's syndrome), no data yet suggest that SPHL is obligatorily tied to any other disease manifestation except thrombocytopenia and oliguria (Park et al., 1989; Thammakumpee et al., 2005; Spichler et al., 2008), or to genetic variations in the human population.

Changes in the underlying health and immune status in the affected human populations may also contribute to the emergence of any disease, including SPHL. For example, Vijayachari et al. (2004, 2008) suggested that having previous exposure, thus seropositivity, reduces the severity of the disease. However, seropositivity does not necessarily equal protective immunity, and it is possible that seropositivity will not be a good predictor of any particular severe disease manifestation over time and in all locations. An additional possibility is that previous subclinical exposure to one serovar may worsen disease caused by a different serovar if the immune system is dysregulated, analogous to the situation for Dengue. Many cases of SPHL are acute, occurring within 1 week of onset of symptomatic illness (the classical "septic" phase), which, if host immunity is found to play a role, might suggest future investigations of possible recall responses or the role of the innate immune system in the development of SPHL. The findings of antibody and complement deposit in the alveoli of humans and guinea pigs suggest a possible role for natural antibodies. It is possible that non-switched IgM antibodies are the pathogenic determinants in these antibody and complement deposits, as it has been demonstrated that the early response to relapsing fever *Borrelia* is dependent on non-T-cell dependent B1b lymphocytes and IgM production (Alugupalli et al., 2003, 2004).

One possibility for future investigation is polymorphisms in human cytokine-encoding genes or their regulatory elements. One example that will be interesting to pursue is sST2, a soluble member of the IL-1 receptor family. Elevated levels of sST2 were associated with severe hemorrhage in a series of severe leptospirosis patients (Wagenaar et al., 2009). Interestingly, sST2 was not induced by leptospires added to peripheral blood, suggesting

that either the cell type responsible for sST2 production was not present, or that the activity of a human or bacterial molecule was inhibited by the heparin used in the blood draw.

Of further interest in future work will be the recognition of *Leptospira* LPS by both TLR2 and TLR4. Previous studies have Implicated TLRs 2, 4, and 5 in human responses to leptospires (Goris et al., 2011). In intact leptospires, the lipid moieties recognized by TLRs 2 and 4, and the flagella classically recognized by TLR5, are not available for recognition, so it is likely that the activation occurs after phagocytosis of the organism by innate defense cells. These receptors apparently also participate in the phagocytosis itself, as previous work has demonstrated the importance of TLR4 in controlling bacterial load and development of severe leptospirosis in mice (Viriyakosol et al., 2006). TLR2 has similarly been implicated in controlling the burden and spread of a variety of other organisms (Wooten et al., 2002; Mancuso et al., 2004; Malik et al., 2006; Abplanalp et al., 2009). The leptospiral protein LipL32, which is common to the pathogenic species, was reported to bind to TLR2 (Hsu et al., 2010), which is a novel activity for both the TLR and for the LipL32, which was previously shown to bind fibronectin (Hoke et al., 2008). These hints of interesting and novel aspects of leptospiral interactions with host defenses will likely provide significant opportunities for research into mechanisms of disease development in leptospirosis.

Severe pulmonary hemorrhagic leptospirosis emergence does coincide to at least some extent with the growth of urban slums in tropical cities. Segura et al. (2005) documented pulmonary involvement in urban but not rural patients in Brazil. There are several possible reasons why this demographic shift may affect the incidence of SPHL. First, the incidence of leptospirosis due to contact with rats and their urine may play a significant role, as rats are maintenance hosts for *L. interrogans* serovars that are particularly pathogenic in humans (Demers et al., 1983, 1985; Vinetz et al., 1996; Adler and de la Pena Moctezuma, 2009). When the disease was more common in rural settings, the serovars and maintenance species were often different.

Second, as has been documented previously, the exposure to leptospires may be higher in the urban slum setting than in the more classical rural setting. In fact, Reis et al. (2008) documented higher risk of leptospirosis, as measured by evidence of exposure by antibody testing, among those living at the bottom of the socio-economic ladder within slums. Ganoza et al. (2006) found higher numbers of human-pathogenic leptospires in urban water sources than in rural settings in Peru. While not all leptospirosis risk studies address rates of particular signs and symptoms, determining whether disease severity correlates with exposure levels or routes may provide important information in future studies. In a different slum population in Brazil, Spichler et al. (2008) did not find a significant difference in leptospirosis survivors vs. non-survivors in environmental exposures.

Delays in treatment, common to poor populations in the developing world, and the inoculum size may be determinants for developing pulmonary hemorrhage (Dolhnikoff et al., 2007). In most endemic areas, other illnesses that are also endemic may cloud diagnosis, contributing to effective treatment delays. However, some cases had rapid development of pulmonary hemorrhage after hospital admission with a brief history (days) of non-specific

illustration. Measures to reduce the leptospiral burden in reservoir animals, and therefore in the environment, as well as investigations of leptospiral gene products and attributes critical to their survival in the environment, can be areas of focus for future work. More readily available health care and diagnostic approaches will also likely reduce the incidence of severe disease in endemic areas.

A critical contribution to understanding SPHL will be the increased recognition of the syndrome and more detailed record keeping by the medical community. This is now a priority for the governments of some of the affected regions, and novel methods of surveillance are being implemented (Spichler et al., 2007, 2008) to assess the frequencies of different manifestations of leptospirosis. Most importantly, increased access to health care for at risk populations will increase the likelihood of survival of severe leptospirosis, including SPHL.

QUESTIONS TO ADDRESS IN FUTURE INVESTIGATIONS

With current limited information on how *Leptospira* species cause infection and disease, it is difficult to distinguish the risk factors and virulence factors that separate SPHL from other leptospirosis manifestations. One priority for future research in leptospirosis will be the identification of specific virulence factors that contribute to the ability of *Leptospira* species to cause infection and disease, which are not equivalent. This will be greatly facilitated by the continuing development of genetic tools for *Leptospira* species and small animal models of human leptospirosis, as well as by genome sequencing. Additional research will need to be done at the epidemiologic level, to determine whether SPHL can be associated with specific environmental or socio-economic factors in the future, as has been published for leptospirosis in general (Reis et al., 2008). Similar investigations will also need to be performed at the patient care level, to determine whether the emergence of SPHL is in part due to better recognition of this manifestation as a possibility in leptospirosis (even in the absence of Weil's syndrome), and to determine whether novel therapeutic approaches can augment antibiotic therapy and supportive care of critically ill patients.

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