Decrease in antithrombin III and prothrombin serum levels contribute to coagulation disorders during leptospirosis

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INTRODUCTION

Leptospirosis, a zoonosis of global concern and considered a re-emerging disease, is caused by pathogenic bacteria of the genus Leptospira. The spirochaete is actively shed in the urine of infected animals, mainly rodents, and human contamination may occur directly or indirectly, via contaminated water or soil, and bacteria may penetrate the host via cuts or abrasions. It occurs in urban settings of developing countries and in countryside (Bharti et al., 2003).

Pathogenic species of the genus Leptospira can cause disease in humans and animals, but the gravity of the disease depends on many issues (Faine et al., 1999; Ko et al., 1999; Plank & Dean, 2000; Levett, 2001). Host infection by pathogenic Leptospira causes an assorted array of clinical symptoms, varying from subclinical to jaundice, renal failure, bleeding and pulmonary haemorrhage, acknowledged as a major – often lethal – clinical sign of leptospirosis (Nicodemo et al., 1997; Marotto et al., 1999; Segura et al., 2005; Maciel et al., 2008).

Abbreviations: DIC, disseminated intravascular coagulation; FXa, factor X-activated; HRP, horseradish peroxidase; IAAX, infected animal number X; LPF, L. interrogans serovar Kennewicki strain Pomona Fromm; MAT, microscopic agglutination test; PLA, plasmin; PLG, plasminogen; uPA, urokinase.

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Pathogenic bacteria of the genus Leptospira are the causative agent of leptospirosis, an emergent infectious disease that affects humans and animals worldwide. Severe forms of the disease in humans include jaundice, multiple organ failure and intense haemorrhage. Up to now, mechanisms associated with the haemorrhage foci are poorly understood. We report in this work that, despite the low levels of antithrombin III in convalescent human serum samples, virulent, culture-attenuated and saprophyte strains of Leptospira are unable to bind and/or degrade this thrombin inhibitor, suggesting an indirect mechanism of pathogenesis. Lower levels of prothrombin were found in serum samples at the onset and convalescent phase of the disease when compared to normal human sera. The concomitant decreased levels of antithrombin III and prothrombin suggest a process of stimulated coagulation, which is corroborated by the increase of prothrombin fragment F1+2 in the serum samples. Data obtained with hamsters experimentally infected with virulent Leptospira interrogans serovars Kennewicki and Canicola strongly point out that haemorrhage is correlated with decreased levels of thrombin inhibitors and prothrombin. Activated coagulation might lead to an overconsumption of coagulation factors ultimately leading to bleeding and organ failure.
clot, a characteristic that might add to the haemorrhagic symptoms in severe leptospirosis. Vieira et al. (2009) have reported that pathogenic leptospires are able to bind plasminogen (PLG) and subvert the host machinery to convert it into its active form, plasmin (PLA), the main fibrinolytic enzyme. PLA-bound leptospires are able to degrade fibrinogen (Oliveira et al., 2013).

Leptospiral haemolysins are reported to be expressed during infection (Zhao & Bao, 2012) but their lytic action upon human erythrocytes is a matter of debate (Zhang et al., 2008; Carvalho et al., 2008). Nevertheless, some of these proteins are secreted and induced pro-inflammatory cytokines (Wang et al., 2002), glycoproteins (Diament et al., 2001), peptidoglycans (Cinco et al., 1996), lipoproteins (Yang et al., 2006a), and outer membrane proteins (OMPs) (Yang et al., 2006a) are capable of promoting host immune response and cytokine secretion.

The interplay between coagulation and inflammation in response to severe infection is becoming evident (Levi et al., 1999, 2003; Wheeler & Bernard, 1999). Systemic activation of coagulation occurs as a consequence of acute inflammation in cases of severe infection or trauma (Esmon et al., 1999, 2003; Wheeler & Bernard, 1999). Nevertheless, some of these activation processes might reach a critical state of disseminated intravascular coagulation (DIC), which eventually will lead to multiple organ failure.

The coagulation cascade results in the cleavage of prothrombin and formation of the enzyme thrombin, which converts the soluble fibrinogen into a tridimensional net of insoluble fibrin (Doolittle, 1984; Weisel, 2005). Antithrombin mostly inhibits thrombin and also has inhibitory effects on other coagulation factors (Levi et al., 2008; Levi & van der Poll, 2008). The anticoagulant property of antithrombin has been extensively demonstrated in vivo (Dickneite, 2008).

In this work, we provide novel evidences that reduced levels of prothrombin and antithrombin in serum samples of leptospirosis patients could be correlated to the bleeding manifestation during this disease, suggesting a state of activated coagulation.

**METHODS**

**Biological components.** Human thrombin, bovine factor X-activated (FXa), antithrombin III from human plasma, the rabbit anti-antithrombin III antibody, human fibrinogen, rabbit anti-prothrombin (anti-F2) antibody, goat horseradish peroxidase (HRP)-conjugated anti-rabbit IgG and BSA were purchased from Sigma-Aldrich.

**Bacterial strains.** Virulent *Leptospira interrogans* serovar Copenhageni strain FIOCRUZ L1-130, *L. interrogans* serovar Canicola strain LO4 and *L. interrogans* serovar Kennewicki strain Pomona Fromm (LPF), pathogenic culture-attenuated *L. interrogans* serovar Copenhageni strain M20 and saprophytic *Leptospira biflexa* serovar Patoc strain Patoc 1 were cultured at 28°C under aerobic and static conditions, in liquid EMJH.

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**Fig. 1.** The effect of leptospirosis human serum samples (pooled) upon exogenous thrombin. (a) Human thrombin (1.25 U ml⁻¹, considered 100% of activity) was incubated for 2 h at 37°C with a pool of leptospirosis serum from the onset (MAT−) and the convalescent (MAT+) phase, diluted ×40 in PBS containing 0.1% BSA. Commercial human normal sera were employed as a control. Ten microlitres of the incubated mixtures were placed onto ELISA plates together with 90 µl human fibrinogen (1 mg ml⁻¹). Experiments were performed three times, each time in triplicate, and the readings were taken at OD₅₉₅ every 2 min to measure fibrin clotting. In the negative control, thrombin was omitted. A concentration curve of thrombin (0–100% of activity) was used to compare the reduction of activity caused by serum samples by linear regression. (b) Inhibition of thrombin after incubation with different serum samples; values are plotted in comparison to the maximal activity used [100% – activity from the curve shown in (a)]. Statistical analysis was performed using one-way ANOVA, followed by Tukey post-test for pairwise comparisons (*P<0.05; **P<0.01).
medium (BD Difco) with 10% (v/v) *Leptospira* enrichment EMJH (BD Difco), which was enriched with l-asparagine (0.015%, w/v), sodium pyruvate (0.001%, w/v), calcium chloride (0.001%, w/v), magnesium chloride (0.001%, w/v), tryptophan (0.03%, w/v), peptone (0.03%, w/v), and meat extract (0.02%, w/v) (Turner, 1970). Virulent leptospire cultures are routinely maintained at the Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo (USP), São Paulo, Brazil, by continuous passage in recently weaned male Golden Syrian hamsters.

**Microscopic agglutination test.** The microscopic agglutination test (MAT) was performed according to methods previously described by Faine et al. (1999). In brief, 22 serovars of species of the genus *Leptospira* were employed as antigens: serovars Australis, Autumnalis, Bataviae, Grippotyphosa, Hardjo, Hebdomadis, Icterohaemorrhagiae, Javanica, Orbignyi, Parahemobartonellae, Pyrogenes, Sejroe, Shewanii, Tarassovi and Wolffi. All the strains were maintained in an EMJH liquid medium at 28°C. A laboratory-confirmed case of leptospirosis was defined by the demonstration of a fourfold increase of the microagglutination titre between paired serum samples. The probable predominant serovar was considered to be the one with the highest dilution that could cause 50% agglutination. The MAT was considered negative when the titre was below 100.

**Effect of human leptospirosis serum samples upon thrombin activity.** Human thrombin (1.25 U ml⁻¹, considered 100% of exogenous thrombin activity) was incubated for 2 h at 37°C with a set (n=16) of human leptospirosis serum samples at the onset (MAT−) or convalescent (MAT+) phase diluted 1x40 in PBS containing 0.1% BSA. Pooled sera, constituted of a normalized mixture of all the samples available, were also employed. As controls, sera from healthy donors or regular commercial human sera (Sigma) were employed at the same dilution (n=8). Ten microlitres of the incubated mixtures were placed onto ELISA plates together with 90 µl human fibrinogen (1 mg ml⁻¹; Sigma), for fibrin clot formation. The experiments were performed in triplicate, and the ELISA plates were read at OD₅₇₀ every 2 min to measure fibrin clotting. In the negative control, thrombin was omitted (0% of exogenous thrombin activity); a concentration curve of thrombin was used to compare the reduction of activity led by serum samples by linear regression. The retained exogenous thrombin activity after incubation was used to calculate the inhibitory effect displayed by the samples by subtraction (100% – thrombin activity). The cutoff value for normal thrombin inhibition was calculated based on the inhibitory effect upon the enzyme displayed by a set of normal human serum samples (n=8) and was established as 35% below the mean of thrombin inhibition. This cutoff value was adapted from protocols employed in our group to define positive responders to leptospiral antigens in immunological assays (Fernandes et al., 2012; Teixeira et al., 2015).

**Hamster infection and evaluation of serum effect upon thrombin.** Four recently weaned male Golden Syrian hamsters were intraperitoneally infected with 10⁵ LLF or LO4, monitored daily for clinical signs of leptospirosis (prostration and loss of appetite) and euthanized when clinical signs of the terminal disease appeared. As controls, hamsters were inoculated with PBS. Animals were bled from the retro-orbital plexus at different time points and the sera stored for analysis. Surveillance of animals to observe jaundice and haemorrhage was performed by technicians, and qualitatively recorded after animal autopsy, by inspection of colour of tissues and the presence of haemorrhagic foci in organs, respectively. The kidneys of animals were extracted, macerated and inoculated in EMJH medium for leptospire isolation. To evaluate the serum effect upon thrombin, the enzyme (1.25 U ml⁻¹) was incubated with the samples from different time points, diluted ×40 and fibrin clot formation was monitored by optical density as previously described, after the addition of a human fibrinogen solution (1 mg ml⁻¹). This assay was based on the experimental data which showed that hamster thrombin inhibitors are able to act upon human thrombin (Mak et al., 1996). The inhibitory effect promoted by hamster serum samples was calculated the same way as for human samples (described above).

**Detection of antithrombin III in human serum samples.** Proteins contained in normal, MAT− and MAT+ pooled or individual human serum samples were fractionated in 12% SDS-PAGE. In each well, 0.25 µl of the sample (previously quantified by Bradford Reagent; Sigma) was applied after boiling at 96°C in denaturing buffer (10% SDS, 50% (v/v) glycerol, 0.5% bromophenol blue and 3% β-mercaptoethanol). Proteins were transferred onto nitrocellulose membranes (Hybond ECL; GE Healthcare) on semidy equipment and the membranes were blocked with 10% non-fat dry milk in PBS containing 0.05% Tween 20 (PBS-T) and then incubated with rabbit anti-antithrombin III antibodies (1:3000 in PBS-T containing 1% milk) for 1 h at room temperature. After washing, detection of bound antibodies was performed by incubation with HRP-conjugated anti-rabbit IgG (1:4000) for 1 h. A control without addition of the primary antibodies was used to verify cross-reaction of the secondary antibody with serum proteins. The protein reactivity was revealed by using the ECL chemiluminescence substrate reagent kit (GE Healthcare); the luminescence generated by the reaction was detected with the aid of Gel Logic 2200 PRO (Equilab). Purified antithrombin III (1 µg) was used as a positive control.

**Western blotting for detection of leptospiral binding to antithrombin III.** Live leptospiral strains (5x10⁵ cells) were washed twice in PBS and then incubated with 100 µl PBS containing 1 µg antithrombin III, for 2 h at 37°C. Reaction mixtures were centrifuged at 5000 g for 15 min, the supernatant collected and the resulting pellet was either resuspended in 100 µl PBS or washed with 1 ml PBS, and then resuspended in 100 µl PBS. Samples (20 µl) were analysed by SDS-PAGE (12%). Gel-fractionated proteins were transferred onto nitrocellulose membranes on semidy equipment, and antithrombin III detection was performed by using specific antibodies as described above.

**Influence of calcium ions upon leptospiral interaction with antithrombin.** Antithrombin III (10 µg ml⁻¹) was incubated with 10⁹ virulent FIOCRUZ L1-130 cells ml⁻¹ in PBS, Tris-NaCl (50 mM Tris-HCl, 100 mM NaCl; pH 8.3) or Tris-NaCl buffer containing 10 mM CaCl₂. After incubation for 16 h at room temperature, incubated solutions were centrifuged (5000 g, 15 min), and both supernatant and pellet collected. The reactions were stopped by addition of 0.25 volumes of SDS-PAGE loading buffer to the samples, followed by boiling for 10 min. Proteins blotted onto nitrocellulose membranes were incubated with anti-antithrombin III antibodies (1:3000), followed by addition of HRP-conjugated anti-rabbit IgG (1:4000).

**Effect of PLA-coated leptospires upon antithrombin III.** Virulent *L. interrogans* serovar Copenhageni strain FIOCRUZ L1-130 (10⁹ leptospires ml⁻¹) were treated with 10 µg PLG, 5 µg urokinase (uPA) or both PLG and uPA (to generate PLA-coated leptospires) in 100 µl PBS. Controls lacking either one or both components were also used. The bacteria were incubated for 2 h at 37°C with PLG, and for an extra 2 h after the addition of uPA. The cells were washed twice with PBS to remove free PLG, uPA or PLA, then leptospires were resuspended in 100 µl PBS containing human antithrombin III (10 µg ml⁻¹), and mixtures were allowed to interact for 16 h at room temperature. Suspensions were separated by 12% SDS-PAGE and then transferred onto nitrocellulose membranes. Detection of components was performed using anti-antithrombin III antibodies (1:3000) as described above.

**Measurement of prothrombin in human and hamster serum samples.** One microlitre of human serum samples (normal, MAT− and MAT+) or hamster serum samples (at different time points of disease) was diluted in 50 µl PBS containing FXa (40 nM) for prothrombin
conversion to thrombin. After incubation for 1 h at 37 °C, 50 µl thrombin chromogenic substrate (N-benzoyl-Phe-Val-Arg-p-nitroanilide hydrochloride, 0.5 mM; Sigma Aldrich) was added to the mixture and 100 µl of the total mixture was placed onto ELISA plates, followed by 40 min incubation at 37 °C. OD readings were taken at 405 nm. Controls lacking FXa, thrombin substrate or serum samples were employed, and when components were omitted, PBS replaced the corresponding volume. Experiments were performed in triplicate. The cutoff for normal prothrombin levels was calculated similarly to the ones for thrombin inhibition, based on the values obtained for a set of normal human serum samples (n=8), and was established as 3σ below the mean of prothrombin level. Detection of prothrombin was also achieved by Western

Fig. 2. The effect of leptospirosis serum samples at the onset and convalescent phase upon exogenous thrombin. (a) Human thrombin (1.25 U ml⁻¹) was incubated for 2 h at 37 °C with a paired set of serum samples at the onset (MAT−, n=16) and convalescent (MAT+, n=16) phase of leptospirosis, diluted 40× in PBS containing 0.1 % BSA. Sera from healthy donors were employed as control (n=8). Ten microlitres of the incubated mixtures was placed onto ELISA plates together with 90 µl human fibrinogen (1 mg ml⁻¹). The experiments were performed in triplicate, and the readings were taken at OD₅₉₅. A concentration curve of thrombin was used to compare the retained exogenous thrombin activity after serum incubation by linear regression, and then, by subtraction, the inhibition upon thrombin. The cutoff value (dashed line) for normal inhibition of thrombin activity was calculated based on the activity of the enzyme after incubation with a set of normal human serum samples and was established as 3σ above the mean of thrombin inhibition. Comparison of mean values (solid lines) was performed using one-way ANOVA, followed by Tukey post-test for pairwise comparisons (*P<0.05; **P<0.01). (b) Values of inhibition of thrombin activity after incubation with each set of paired serum samples. Student’s paired t-test was used for comparison between MAT− and MAT+ values (*P<0.05).
Fig. 3. Leptospira infection causes a decrease in antithrombin III serum levels. Proteins contained in 0.25 µl pooled normal, MAT– and MAT+ human serum samples were fractionated in 12% SDS-PAGE. (a) Membranes were Ponceau stained for control of total loaded protein. (b) Membranes were blocked with skimmed milk proteins, and then incubated with rabbit anti-antithrombin III antibodies (1:3000 in PBS-T containing 1% milk) for 1 h at room temperature. After washing, detection of bound antibodies was performed by incubation with HRP-conjugated anti-rabbit IgG (1:4000) for 1 h. Asterisk denotes reactivity between the secondary antibody and serum proteins. The protein reactivity was revealed by use of the ECL reagent kit chemiluminescent substrate. Purified antithrombin III (1 µg, AT) was employed as a positive control. (c) Antithrombin contained in individual leptospirosis human serum samples was evaluated. Normal and paired leptospirosis serum samples at the onset (MAT–) and convalescent (MAT+) phase were included. Numbers refer to paired samples used in Fig. 2(b). Purified antithrombin III (1 µg, AT) was employed as a positive control.
blotting, as previously described. In brief, 0.25 µl human serum samples were run through SDS-PAGE, and proteins transferred onto nitrocellulose membranes. Detection of prothrombin and prothrombin activation fragment (F1+2) was performed by using rabbit anti-F2 antibodies (1:3000, in PBS-T 1% milk), followed by HRP-conjugated anti-rabbit IgG (1:4000, in PBS-T 1% milk).

Statistical analysis. All experiments were performed at least twice. Representative Western blotting experiments are shown in the figures. All results are expressed as the mean±SD. Statistical analysis for multiple comparisons was performed using one-way ANOVA, followed by Tukey post-test for pairwise comparisons, and P<0.05 was considered statistically significant. Student’s paired t-test was also used.

Ethics statement. All animal studies were approved by the Ethical Committee for Animal Research of Instituto Butantan, São Paulo, Brazil under protocol 1025/13. The Committee in Animal Research of Instituto Butantan adopts the guidelines of the Brazilian College of Animal Experimentation. Human serum samples from patients with confirmed leptospirosis were obtained from the Serum Collection of Instituto Adolfo Lutz, São Paulo, Brazil, and were donated for research purposes only. The Ethical Committee for Human Research of ICB/Universidade de São Paulo rules that this work does not involve procedures regulated by CONEP/Brazil no. 466/2012, and official approval was, therefore, not required.

RESULTS

Human leptospirosis serum samples display low inhibitory effect upon exogenous thrombin activity

Taking into account the coagulation disturbance found in patients with leptospirosis, we decided to evaluate whether serum samples of confirmed individuals might affect the activity of exogenous added thrombin. After incubation of the enzyme with a pool of serum (described in Methods) from the convalescent phase (MAT+), almost no inhibition of enzyme activity was observed, while at the onset of the disease (MAT−), approximately 50% of enzyme activity was detected (Fig. 1a, b). In contrast, sera from healthy donors fully inhibited thrombin activity (Fig. 1a, b). These data suggest that some pathophysiological mechanisms occur during leptospirosis leading to a decrease in thrombin inhibition, starting at the onset of the disease with continuous decrease at the convalescent phase. The decrease of inhibition upon exogenous thrombin activity observed was statistically significant for both MAT− and MAT+ phases, when compared to normal serum, suggesting a decrease of circulating levels of thrombin inhibitors during the evolution of the disease.

Evaluation of thrombin inhibition displayed by individual leptospirosis serum samples

We employed a set of 16 paired serum samples (MAT− and the corresponding MAT+) for the evaluation of individual serum effect upon thrombin activity. The calculated cutoff value based on normal serum samples was 81% of thrombin inhibition. The data obtained with MAT− samples showed that 68% of individuals had abnormal levels of thrombin inhibitors and that the mean value for thrombin inhibition was 60%, which was statistically significant in comparison to normal serum samples (P<0.05). In MAT+ samples, all serum samples had decreased thrombin inhibitors in serum, as represented by the low mean of thrombin inhibition of approximately 30% (Fig. 2a). The mean value obtained for normal samples was 93% of enzyme inhibition. Comparison of MAT+ to normal and MAT− serum samples showed statistical significance of thrombin inhibition values (P<0.01 and P<0.05, respectively). These data are consistent with the representative mean values obtained with the pooled samples (Fig. 1), and point to a decrease in thrombin inhibitors in serum during leptospirosis.

The dispersal of the values obtained for each set of paired samples is depicted in Fig. 2(b); the most frequent profile (56%) was still high serum levels of thrombin inhibitors at the onset and then a decrease of these inhibitors. In some cases, thrombin inhibitor levels were already low at the initial phase (25%), which might explain the divergent response among different patients. Indeed, in one case, the patient showed a tendency for normalization of thrombin inhibitor levels. No correlation between thrombin inhibition and MAT titres was observed (not shown).

Markedly low levels of antithrombin III are found in convalescent leptospirosis serum samples

Some authors have suggested the involvement of disseminated intravascular coagulation (DIC) in the course of leptospirosis (da Silva et al., 1995; Chierakul et al., 2008). Moreover, one clinical feature of this scenario is the reduced serum levels of antithrombin III (Levi et al., 2003). Thus, we decided to evaluate whether the low levels of this specific inhibitor could be responsible for the decrease in thrombin inhibition observed with the exogenous enzyme. We assayed leptospirosis human serum samples for the presence of antithrombin III in comparison to the levels found in normal human serum, by Western blotting using specific antibodies.

The total amount of protein loaded per lane was similar (Fig. 3a); thus the results show that the lowest levels of antithrombin III (approximately 58 kDa) were found in MAT+, followed by MAT− and normal serum samples (Fig. 3b). These data comply with our previous results that showed the lowest inhibition upon activity of exogenous thrombin detected with MAT+ and MAT− leptospirosis samples (see Figs 1 and 2). Protein bands, of approximately 45 kDa, observed in Western blotting (denoted by * in Fig. 3b) correspond to unspecific cross-reactivity of serum samples with the secondary antibody (not shown). To further corroborate these interesting findings, individual paired leptospirosis human serum samples, employed in the thrombin inhibition assay shown in Fig. 2(b), were accessed for the presence of antithrombin III molecule. As depicted in Fig. 3(c), a good correlation was observed regarding the thrombin inhibition profile exhibited by individual serum samples and the presence of antithrombin III molecule.
Our results indicate that there is a reduction of antithrombin III serum levels during the progression of the disease, which may indicate an exacerbated consumption of coagulation factors due to a disseminated coagulation (Levi et al., 1999) and/or binding/degradation of antithrombin III by leptospiral strains.

Leptospiral strains are unable to bind to antithrombin III

Once decreased levels of antithrombin were detected in convalescent sera of leptospirosis patients, we decided to further study if this decrease was due to a direct effect of spirochaetes, by either binding or degradation. We took advantage of different available strains of leptospires to infer whether the binding, if occurring, could be correlated to virulence. Three virulent strains of L. interrogans (LPF, FIOCRUZ L1-130 and LO4), one culture-attenuated strain of L. interrogans (M20) and one saprophyte strain of L. biflexa (Patoc 1) were incubated with antithrombin in a soluble phase manner, as described by Fernandes et al. (2015). After 2 h interaction, cultures were centrifuged, and pellets and supernatants loaded for SDS-PAGE: supernatant (SN), resuspended pellet (P) and resuspended pellet after washing with 1 ml of buffer (P*). Fractionated proteins were transferred onto a nitrocellulose membrane and probed by using anti-antithrombin antibodies (1:3000) and HRP-conjugated anti-rabbit IgG (1:4000), followed by ECL reagent kit chemiluminescence substrate. "AT" refers to the antithrombin molecule. (b) Antithrombin degradation was evaluated in different buffers after 16 h: PBS (1), Tris-NaCl (2) and Tris-NaCl plus 10 mM CaCl$_2$ (3). Both supernatant and pellet were obtained as described in (a). (c) Virulent L1-130 strain (10$^9$ leptospires ml$^{-1}$) was treated in 100 µl PBS with the addition of 10 µg PLG, 3 U uPA, or both PLG and uPA (generating PLA$_2$-coated leptospires). Controls lacking either one or both components were also used. Leptospires were then resuspended in 100 µl PBS/0.1% BSA, containing human antithrombin (10 µg ml$^{-1}$), and mixtures were allowed to interact for 16 h at room temperature. Suspensions were separated by 12% SDS-PAGE and then transferred onto nitrocellulose membranes. Detection of components was performed as previously described with anti-antithrombin (1:3000) and the arrow indicates a degradation band.
able to promote only a slight degradation of this component even in the presence of an excess of Ca$^{2+}$ ions. The data confirm that leptospires, independently of the buffer condition, exert no effect upon antithrombin III. A similar result was obtained with the *L. interrogans* virulent LO4 strain (not shown).

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**Fig. 5.** Low prothrombin levels in human leptospirosis serum samples are related to *in vivo* prothrombin activation. (a) One microlitre of pooled human serum samples (normal, MAT− and MAT+) was diluted in 50 µl PBS containing 0.25 µl FXa. After 1 h incubation at 37 °C, 50 µl thrombin chromogenic substrate were added to the mixture and 100 µl total mixture were placed onto ELISA plates, followed by 20 min incubation at 37 °C; readings were taken at 405 nm. Controls lacking FXa, thrombin substrate or serum samples were employed, and when components were omitted, volumes were replaced with PBS. Experiments were performed in triplicate. Detection of prothrombin was also performed by Western blotting (b); left panel: Ponceau-stained membrane for total protein loading/transferred control; right panel: detection of prothrombin (PT) and prothrombin activation fragment (F1+2) was performed using anti-F2 antibodies (1 : 3000) followed by HRP-conjugated anti-rabbit IgG (1 : 4000). The asterisk (*) refers to unspecific cross-reaction with the secondary antibody. (c) A set of 16 paired human serum samples were employed for indirectly detecting prothrombin; the latter upon FXa activation is converted into thrombin, which in turn acts on the chromogenic substrate. The cutoff value (dashed line) was calculated based on the indirectly measured prothrombin levels from a set of normal human serum samples (*n*=8) and was established as 3sd below the mean prothrombin normal level; 68 and 56% of prothrombin levels, below the cutoff value, were detected with MAT− and MAT+ serum samples, respectively. Comparison of mean values (solid line) was performed using one-way ANOVA, followed by Tukey post-test for pairwise comparisons (**P<0.01).
Virulent leptospires promote a modest degradation of antithrombin III by using PLG/PLA system

Our group has reported that leptospires are capable of capturing human PLG and subvert the human machinery to produce its active form, PLA (Vieira et al., 2009). We thus decided to assess if leptospire-bound PLA could degrade antithrombin III, and therefore contribute to the low levels observed in leptospirosis serum samples. After incubation with PLG and/or its activator, uPA, membrane-bound PLA promoted only partial degradation of the component after 16 h of incubation (Fig. 4c, indicated by an arrow). Degradation was not observed when at least one of the reaction components was missing. We believe that this modest degradation alone is not sufficient to explain the decrease in the antithrombin III serum levels.

Low prothrombin serum levels during leptospirosis might be correlated to bleeding

It is well known that activation of the coagulation cascade by micro-organisms and inflammation leads to an overconsumption of coagulation factors, including coagulation inhibitors (Esmon et al., 1999; Levi et al., 1999). Hence, we set out to examine whether there is a correlation between low antithrombin III and prothrombin levels in serum, since it has been reported that leptospirosis patients display elevated prothrombin time (Esen et al., 2004; Chierakul et al., 2008).

Human normal and leptospirosis sera, at the onset and convalescent phase, were employed as a source of prothrombin to generate thrombin, detected through the measurement of its activity. After incubation of sera with FXa, which converts prothrombin to thrombin, a chromogenic substrate was added, to measure thrombin activity and, indirectly, prothrombin levels. The results show that only normal human serum displayed an elevated and statistically significant (P<0.01) thrombin activity, which could be directly correlated to the high levels of prothrombin in this sample (Fig. 5a). Pooled human leptospirosis serum at the onset (MAT−) and at the convalescent (MAT+) phase did not show any significant thrombin activity, indicating that prothrombin levels are drastically reduced due to *Leptospira* infection. Controls lacking at least one of the components did not present any significant signal at the time of analysis, except for the treatment composed by normal serum and substrate, which showed an increase of signal, possibly due to the activation of the intrinsic pathway of the coagulation system.

To further corroborate these interesting findings, a Western blotting experiment was performed to assess the presence of prothrombin in human serum samples, by using anti-F2 antibodies. Ponceau-stained membrane shows that similar amounts of proteins were loaded/transferred (Fig. 5b, left panel). A prothrombin band (~72 kDa) was detected only in normal human serum samples, and accordingly, no prothrombin F1+2 fragment (resulting from prothrombin in vivo activation) was observed (Fig. 5b, right panel). As expected, a prothrombin band was not found either in leptospirosis MAT−, or in MAT+ serum samples (Fig. 5b). Moreover, the prothrombin F1+2 fragment was detected mainly in MAT+ serum samples, suggesting that activation of the coagulation cascade occurs during leptospirosis progression. Although the F1+2 fragment was not detected in normal human serum samples, it is reasonable to assume that some amount of this molecule is present, as a result of normal blood clotting, but in a concentration below the sensitivity of our experiment.

We further decided to evaluate prothrombin levels in a set of 16 paired human serum samples. We used FXa to transform prothrombin to thrombin and measured thrombin activity upon addition of chromogenic substrate; this will indirectly assess prothrombin levels (Fig. 5c). The cutoff value (dashed line) was calculated based on the indirectly measured prothrombin levels from a set of normal human serum samples (n=8) and was established as 3SD below the mean value. The results show that 68 and 56% of prothrombin levels, below the cutoff value, were detected with MAT− and MAT+ leptospirosis serum samples, respectively (Fig. 5c).

Low thrombin inhibition and prothrombin levels found in hamster serum samples are correlated with bleeding

In view of our findings, we decided to evaluate thrombin inhibition and prothrombin levels in serum samples of infected animals (IAx) during the progression of the disease. Hamsters were experimentally infected either with virulent *L. interrogans* serovar Kennewicki strain Pomona Fromm (LPF, slightly haemorrhagic) or *L. interrogans* serovar Canicola strain LO4 (LO4, highly haemorrhagic) and monitored until clinical signs of terminal disease emerged. Serum was collected at different time points of infection, and animals inoculated with saline solution were used as controls (C1 and C2). Death profiles of IAX in days post-infection for each strain are depicted in Fig. 6(a). Animals infected with LPF exhibited a very heterogeneous evolution of the disease; IA4 died earlier than the others and presented varied haemorrhagic foci, blood in urine, slight jaundice, and haemolysis was found in its serum. IA1 died at the beginning of day 10 and did not present indications of haemorrhage. IA2 died at day 12, or at the beginning of day 13, and when serum was collected at day 11, haemorrhage was observed. IA3 showed moderate symptoms at day 11, but it recovered from the disease, being completely healthy at the end of observation time, at day 21. This heterogeneity of outcomes due to infection with LPF has been routinely observed in our laboratory.

Animals experimentally infected with LO4 strain had a more uniform evolution of leptospirosis (Fig. 6a); IA1, IA2 and IA4 died at day 10, and IA3 died at day 8. It is worth mentioning that all animals presented severe bleeding, blood in
Fig. 6. Bleeding during *Leptospira* infection in hamsters is strongly related to decreased thrombin inhibitors and prothrombin serum levels. (a) Evolution of the disease, evaluated per days post-infection, shown by animals infected (IA-X) with either LPF or LO4. (b) Thrombin (1.25 U ml\(^{-1}\)) was incubated for 2 h at 37 °C with hamster serum samples, diluted × 40 in PBS containing 0.1 % BSA, at day 0 (D0) and at the last day of analysis (D\(^*\)). Ten microlitres of the incubated mixtures was placed onto ELISA plates together with 90 µl human fibrinogen (1 mg ml\(^{-1}\)) and OD\(_{595}\) readings were taken every 2 min to measure fibrin clotting, which was used to calculate the inhibitory effect upon the enzyme. Data are representative of three independent experiments, each time performed in triplicate; values displayed by D\(^*\) sera were compared to those displayed by D0, by two-tailed t test (**P < 0.01; ***P < 0.001). (c) One microlitre of hamster serum samples (day 0 and last day of analysis, D\(^*\)) was diluted in 50 µl PBS containing 0.25 µl FXa. After 1 h incubation at 37 °C, 5 µl thrombin chromogenic substrate was added to the mixture and 100 µl total mixture was placed onto ELISA plates, followed by 40 min incubation at 37 °C, and readings were taken at OD\(_{405}\). Controls lacking substrate were employed as blank. Experiments were performed three times and each time in triplicate. OD\(_{405}\) values obtained with serum samples at D\(^*\) were compared to those at D0, by two-tailed t-test (*P < 0.05; **P < 0.01; ***P < 0.001).
urine and jaundice. All animals in the experiment were culture-positive for leptospires after kidney isolation. Serum from non-infected animals (C1 and C2) exhibited the same high inhibitory effect upon exogenous thrombin, at the first and last days of observation (D0 and D*, respectively, corresponding to day ‘*’ in Fig. 6a) (Fig. 6b). LPF-infected animals showed heterogeneous serum inhibition of thrombin, which may reflect the different death profile observed (Fig. 6a). IA2 and IA4, which died with haemorrhagic manifestations, presented less inhibition of the enzyme at the last day of infection (P<0.01 and 0.001, respectively). Serum of IA1 and IA3 did not show lesser inhibition of thrombin; IA1 did not present signals of haemorrhage and IA3 survived the infection. It is important to mention that serum of IA3 at day 21 presented as much thrombin inhibition as the control animals (C) (data not shown).

All animals infected with the haemorrhagic strain LO4 revealed less thrombin inhibition at the last day of analysis (D*) (Fig. 6b). These data suggest that a decrease in thrombin inhibitors might contribute to hemorrhage in severe cases of leptospirosis. We were prompted to investigate if a low level of thrombin inhibitors in serum, possibly antithrombin III, as demonstrated in human serum, was correlated with a decrease in prothrombin levels. Serum samples of animals were incubated with FXa and then a thrombin chromogenic substrate was added. We employed a control lacking substrate to discount the basal colour due to serum, especially from animals presenting jaundice. Animals that exhibited a reduced inhibition of exogenous thrombin also presented low prothrombin levels, suggesting an imbalance and consumption of coagulation factors (Fig 6c, LPF: IA2 and IA4; LO4: IA1–4). These data are in agreement with the ones obtained with human serum samples.

A strong correlation between lower levels of thrombin inhibitors, prothrombin and bleeding in the hamster model was found, indicating an activation of the coagulation cascade in response to Leptospora infection.

**Proposed schematic mechanism for haemorrhagic manifestations during severe leptospirosis**

After bacterial penetration into the bloodstream, leptospires disseminate to reach target organs. During this leptospiremia phase, molecular patterns and proteins from the pathogens begin to be recognized by the host immune system and the coagulation factors such as prothrombin, and inhibitors such as antithrombin III, are presented in normal and physiological levels. Factors now present in serum, due to inflammation, initiate the coagulation cascade, resulting in a systemic coagulation, which leads to an overconsumption of prothrombin, resulting in large amounts of its activation product, namely F1+2. The antithrombin III is consumed as large amounts of thrombin are formed. Finally, when endothelial damage occurs, due to inflammation itself or some factor secreted by leptospires (now in the kidneys, leptospiururia phase), the lack of coagulation factors, especially prothrombin, causes a decrease in fibrin clot formation, ultimately leading to bleeding (Fig. 7).

**DISCUSSION**

Although haemorrhage in leptospirosis was first observed by Weil in 1886 (Faine *et al*., 1999), its pathophysiology is still not clearly explained, mainly regarding the origin and mechanisms of bleeding. In theory, bleeding may be the result of a defect in the primary haemostasis or an imbalance in secondary haemostasis by exhaustion of coagulation proteins because of increased coagulation or by stimulated fibrinolysis (Wagenaar *et al*., 2007).

Infection-associated stimulation of the coagulation cascade may lead to a varied spectrum of clinical effects, from a clinically unimportant increase in laboratory markers to severe thrombo-haemorrhage syndromes, such as DIC (van Gorp *et al*., 1999). The pathophysiology of sepsis-associated DIC is very complex and has been exhaustively examined. The major occurrence is the systemic inflammatory reaction to the infectious agent (Tsujimoto *et al*., 2008; Cinel & Opal, 2009). This syndrome may be promoted by the microorganism expressing unique cellular components, known as pathogen-associated molecular patterns (PAMPs).

A study by Higgins & Cousineau (1977) demonstrated that thrombocytopenia, increase of prothrombin and coagulation times and reduction of plasma fibrinogen, factor V and factor VIII in guinea pigs infected with Leptospira were due to DIC. Later, pathological and haematological analyses of guinea pigs experimentally infected with Leptospira indicated that the thrombocytopenia did not correlate with the existence of DIC (Yang *et al*., 2006b). Another study, in the same animal model by Silva *et al.* (1995), concluded that the clinical-laboratory picture in most of the infected animals is compatible with the histopathological observation of DIC.

Under physiological conditions, the surface of endothelial cells expresses various components of the anticoagulant pathways, which are rapidly and significantly decreased in the sepsis-induced DIC process (Aird, 2005). To our knowledge, this is the first time that leptospirosis patients are reported to exhibit less inhibition of thrombin, especially in the convalescent phase of the disease, with this finding related to decreased serum levels of antithrombin III. Moreover, some authors suggest that a rapid depletion of antithrombin is associated with a poor prognosis in humans regarding bleeding manifestations (Levi, 2010). Accordingly, we observed a strong correlation between intense haemorrhage and decreased thrombin inhibition shown by serum from infected hamsters. The reduction of antithrombin in leptospirosis patients has been shown previously (Wagenaar *et al*., 2010).

The decrease in serum levels of antithrombin might be a result of two events: Leptospira strains could bind to and degrade this component, or an activation of coagulation *in vivo* would lead to an exacerbated consumption of
coagulation factors, as well as coagulation inhibitors. We tested the ability of different leptospiral strains to bind antithrombin on their surface and found that none of the strains, neither virulent, culture-attenuated nor saprophyte, could bind the component. Direct degradation by virulent leptospires, in the presence of calcium ions, or indirectly, via PLG/PLA system (Vieira et al., 2009) were very modest and, presumably, insufficient to explain the decreased levels of antithrombin. This reduction was especially noticeable in the convalescent phase, in which no leptospires are found in blood, suggesting an indirect effect promoted by Leptospira infection.

Acute inflammation, a response to severe infection or trauma, promotes a systemic activation of the coagulation system (Esmon et al., 1999; Levi et al., 1999) and pro-inflammatory cytokines play a pivotal role in this activation (Levi et al., 2003). TNF-α activated the coagulation system via the tissue factor pathway in healthy volunteers (Bauer et al., 1989; van der Poll et al., 1990). Tajiki & Salomao (1996) measured plasma levels of TNF-α in leptospirosis patients and found the TNF-α level to be related to disease severity. These studies suggested that TNF-α level could be a useful marker for poor prognosis of the disease. It has also been shown that leptospiral peptidoglycan induces the release of TNF-α from human monocytes (Cinco et al., 1996).

Similar to sepsis, human leptospirosis was reported to be associated with high plasma levels of inflammatory cytokines such as TNF-α, IL-6, IL-8, IL-1β and IL-12 (Matsui et al., 2011; Wang et al., 2012; Reis et al., 2013). Indeed, the production of inflammatory cytokines such as TNF-α by CD4+ T-cells after stimulation with antigens of Leptospira was highest in patients with severe disease (Volz et al., 2015).

Furthermore, it has been shown that the major protein LipL32 induces inflammatory responses through the Toll-like receptor 2 pathway in human renal cells (Lo et al., 2013).
Activation of coagulation would lead to, besides a reduction of antithrombin III, an overconsumption of coagulation factor. Chierakul et al. (2008) showed that patients with leptospirosis had longer prothrombin times, which may be due to a decrease or inactivation of coagulation factors in plasma. Moreover, Wagenaar et al. (2010) observed coagulation parameters in plasma of patients with leptospirosis compatible with an activated coagulation. We assayed for the presence of prothrombin in human serum samples by different approaches. Chromogenic experiments showed that normal human serum presents high levels of prothrombin, measured after FXa-mediated activation. As the disease progresses, drastically reduced prothrombin levels are found, especially in convalescent serum samples, which showed less substrate cleavage. Furthermore, Western blotting of these samples showed very interesting results: the prothrombin band is only found in normal human serum, and prothrombin activation fragment F1+2, generated from prothrombin in vivo activation, was found exclusively in leptospirosis sera, being more prominently in the convalescent phase, indicating an activation of the coagulation system. The increase in the serum levels of prothrombin activation fragment F1+2 was also found by Chierakul et al. (2008).

Upon Leptospira infection, hamsters that presented features of intense haemorrhage (and, as shown, reduced thrombin inhibitors) also had lower prothrombin levels in the last days of disease, strengthening the activation of the coagulation system, most probably, due to inflammatory cytokines (Matsui et al., 2011). The host response to virulent Leptospira might generate an initial exacerbated coagulation, which would ultimately lead to a lack of coagulation factors and inhibitors during the course of the disease. Accordingly, when mononuclear cells were stimulated in vitro by a virulent or non-virulent strain of L. interrogans serovar Icterohaemorrhagiae, different coagulant activity was observed, being more prominent with the virulent one (Miraglia & Fumara, 1983). This effect was not observed when FVII-depleted plasma was used, suggesting a tissue-factor-dependent mechanism. Indeed, it has been shown that tissue factor is elevated in leptospirosis sera (Vieira et al., 2016).

This work brings new pieces of evidence to the complex puzzle that is the haematopathology caused by leptospirosis. All results have pointed to the activation of the coagulation system, resulting in a decrease in antithrombin III and prothrombin levels. Once leptospires are unable to bind antithrombin and prothrombin (Fernandes et al., 2015), it seems that reductions of these components are due to an indirect effect of bacteria. Future studies aiming to elucidate the mechanism by which the coagulation is activated are currently being carried out.

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