Diminished coagulation capacity assessed by calibrated automated thrombography
during acute Puumala hantavirus infection

Running head: Decreased endogenous thrombin potential in hantavirus infection

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Conflicts of interests: none.
Abstract

Objectives

Coagulation abnormalities are associated with Puumala virus-induced hemorrhagic fever with renal syndrome (PUUV-HFRS). We evaluated the coagulation capacity of plasma during acute PUUV-HFRS by measuring thrombin generation using calibrated automated thrombography (CAT®).

Material and Methods

The study cohort comprised of 27 prospectively collected, consecutive, hospital-treated patients with acute PUUV infection. Blood samples were drawn in the acute phase and at the control visit approximately 5 weeks later. To evaluate thrombin generation, the lag time of initiation, endogenous thrombin potential (ETP), and peak and time to peak thrombin concentration were assessed by CAT® in platelet poor plasma without corn trypsin inhibitor. Plasma levels of D-dimer, fibrinogen and prothrombin fragments (F1+2) were also evaluated.

Results

When the acute phase was compared with the control phase, ETP was decreased (median 1154 nM/min, range 67-1785 vs. median 1385 nM/min, range 670-1970; p<0.001), while the lag time was prolonged (median 3.8 minutes, range 2.1-7.7 vs. median 2.9 minutes, range 2.0-4.1; p<0.001). Low ETP correlated with low peak thrombin concentration (r=0.833, p<0.001). Prolonged time to peak associated with the lag time (r=0.78, p<0.001). ETP was associated with thrombocytopenia (r=0.472, p=0.015) and weakly with fibrinogen level (r=0.386, p=0.047). The measured CAT® parameters did not associate with D-dimer and F1+2 levels.

Conclusions

Decreased ETP together with low peak and prolonged lag time indicate decreased plasma potential for thrombin generation in vitro. Together with low platelet count and enhanced fibrinolysis this further refers to altered blood coagulation and increased propensity toward bleeding in acute PUUV-HFRS.
**Keywords:** coagulation; Calibrated automated thrombography; thrombin; hantavirus; platelet; fibrinolysis

**Abbreviations:** ADAMTS13, a thrombospondin type 1 domain; APTT, activated partial thromboplastin time; AT, antithrombin; CAT®, calibrated automated thrombography; CRP, C-reactive protein; ETP, endogenous thrombin potential; F1+2, prothrombin fragments; HCPS, hantavirus cardiopulmonary syndrome; HFRS, hemorrhagic fever with renal syndrome; LT, lag time; PC, protein C; PPP, platelet poor plasma; PS, protein S free antigen; PT, prothrombin time; PUUV, Puumala virus; TAFI, thrombin activatable fibrinolysis inhibitor; TF, tissue factor; TG, thrombin generation; tPA, tissue plasminogen activator; TT, thrombin time; tt Peak, time to peak.
Introduction

Hantaviruses are the cause of two disease entities, hemorrhagic fever with renal syndrome (HFRS) in Europe and Asia, and hantavirus cardiopulmonary syndrome (HCPS) in North and South America. Puumala hantavirus (PUUV) causes mild HFRS, also called nephropathia epidemica in Europe [1, 2]. PUUV-HFRS is characterized by thrombocytopenia and coagulation abnormalities, acute kidney injury and capillary leakage [1-3]. Petechiae, epistaxis, hematuria and conjunctival bleedings are common [1]. Hemorrhagic gastropathy is observed in all PUUV-HFRS patients in gastroscopy [4]. Severe and fatal hemorrhages of pituitary gland, kidneys, heart, liver, lungs and peritoneal cavity have been described [5, 6]. The risk for cardiovascular disease has also been linked with PUUV-HFRS [7]. Disseminated intravascular coagulation has been encountered in severe cases [8, 9].

Hantaviruses target vascular endothelial cells via β3 integrin receptor and adhere quiescent platelets to the endothelium, thus contributing to vascular permeability and thrombocytopenia [10]. Through interactions with hantavirus, platelets and endothelium, alterations in the coagulation system occur. Previous studies imply enhanced thrombin formation, as evaluated by shortened prothrombin time (PT) and thrombin time (TT), and overall increase in prothrombin fragments 1+2 (F1+2), and decreased levels of natural anticoagulants, antithrombin (AT), protein C (PC) and protein S free antigen (PS) [8, 11]. A study with PUUV infected human umbilical vein endothelial cells suggests increased tissue factor (TF) activity [12]. Fibrinolysis is activated as indicated by increased concentrations of fibrin degradation products, D-dimer and tissue plasminogen activator (tPA) [8, 13]. Platelet ligands are altered, and ADAMTS13 activity is decreased [14].

Thrombin is the key enzyme during coagulation leading to the conversion of fibrinogen to fibrin and clot formation. Thrombin generation (TG) assays are useful indicators of the overall plasma coagulability, in contrast to the conventional coagulation tests that mainly assess individual factors or a part of the coagulation pathway. Calibrated automated thrombography (CAT®) measures in vitro TG of plasma by continuous cleavage of a fluorogenic substrate, thus expressing the overall haemostatic potential [15]. CAT®
is applied in research of vascular thrombosis, bleeding disorders and monitoring of anticoagulant treatment [16]. To our knowledge, studies on TG by CAT® in hantavirus infections are yet lacking.

Both hemorrhagic and thrombotic events have been associated with PUUV-HFRS, but the underlying mechanisms of alterations in coagulation system are not well defined. Therefore, we aimed to evaluate the plasma coagulation capacity in PUUV-infected patients by measuring TG by CAT®. The goal was to describe how TG is altered during the acute phase of infection, and further determine the possible hypo- or hypercoagulability associated with hantavirus infection. We also sought to investigate the possible associations of CAT® assay with the tests applied to measure thrombin formation and fibrinolysis in clinical practice and variables depicting disease severity of acute PUUV-HFRS.

Material and Methods

Ethics statement

All patients were recruited and enrolled after providing a written informed consent. The study protocol was approved by the Ethics Committee of Tampere University Hospital. The study was conducted according to the principles expressed in the Declaration of Helsinki.

Patients

The study was carried out in Tampere University Hospital, University of Tampere, Helsinki University Hospital and University of Helsinki. All patients came from the Pirkanmaa area and were hospitalized at Tampere University Hospital due to serologically confirmed acute PUUV-HFRS [17] during the period from October 2010 to February 2014.

Twenty-seven prospectively collected, consecutive patients (17 males) with acute PUUV-HFRS were included. Their median age was 50 years (range 21-67 years). None of the subjects used anticoagulant or immunosuppressive therapy. Two patients used an anti-platelet drug (acetylsalicylic acid).
Clinical and laboratory data

The following variables were recorded: the number of days from the onset of fever before the acute-phase study samples were collected, the length of hospital stay (days), signs of bleeding symptoms (yes/no), thromboembolic complications (yes/no), need for transient hemodialysis treatment (yes/no), and maximum gain in weight (kg). Complete blood count, plasma C-reactive protein (CRP) and plasma creatinine were measured according to clinical need. The laboratory analyses were carried out at the Laboratory Centre of Pirkanmaa Hospital District using standard methods.

Methods

The study design was longitudinal with two time-points of blood draw for CAT®. The acute phase samples (n=27) were taken median 7 days (range 4-12 days) from the onset of fever. Control samples (n=23) were taken at the follow-up visit, median 43 days (range 38-76 days) from the onset of fever. The blood count was assessed in the acute and control phase of CAT® study days, and the lowest platelet count during the hospital stay was recorded.

CAT® analyses and plasma measurements of fibrinogen, F1+2 and D-dimer were carried out in Clinical Chemistry coagulation laboratory (HUSLAB Laboratory Services, Helsinki University Central Hospital, Finland). D-dimer (Tina Quant D-Dimer®, Roche Diagnostics, Mannheim, Germany) and fibrinogen (Multifibren U® Siemens Healthcare Diagnostics) levels were determined according to manufacturer’s recommendations. F1+2 were measured by an enzyme immunoassay (Enzygnost® F1+2, monoclonal, Siemens Healthcare Diagnostics). The reference values for D-dimer were ≤ 0.5 mg/l, fibrinogen 1.7-4.0 g/l and F1+2 69-229 pmol/l.

Measurement of thrombin generation by CAT®

TG was measured using CAT® (Diagnostica Stago) with the Stago PPP reagent (tissue factor 5 pM and phospholipids 4 μM) without corn-trypsin inhibitor. The lag time of the initiation of TG (LT, min), the endogenous thrombin potential (ETP; the area under the curve; nM thrombin x time), peak (maximum thrombin concentration, nM) and time to peak (tt Peak, min) were measured and recorded according to the
manufacturer's instructions. Blood samples were collected into sodium citrate anticoagulant (3.2%; 109 mM) tubes according to the local sampling protocol as part of hospital routine, and centrifuged (at 2500 g for 15 min). The PPP was separated within 2 hours and stored at -80 °C before analysis.

Statistics

All continuous, skewed variables were determined as medians and ranges. The associations between TG markers and clinical and laboratory variables were evaluated for continuous data by Spearman rank correlation coefficient. To analyze the changes between the acute and control phase, Wilcoxon-signed rank test was used for pairwise comparisons. The level of significance was set at p value 0.05 (2-tailed). Statistical analyses were performed with IBM SPSS Statistics for Windows version 22.0 (Armonk, NY, USA).

Results

Clinical and laboratory findings

All 27 patients suffered from clinically typical and serologically confirmed PUUV-HFRS [17]. The clinical and laboratory findings of the patients are shown in Table 1. Mild bleedings were reported in eight patients including nasal and conjunctival hemorrhages, petechiae, hemoptysis and melena. There were no thromboembolic events recorded. None of the patients needed transient hemodialysis treatment.

The acute phase median platelet count was 68 x 10⁹/l (range 8-222 x 10⁹/l), CRP 57 mg/ml (10-178 mg/ml), hemoglobin 139 g/l (120-177 g/l), hematocrit 0.40 (0.34-0.49) and creatinine 126 µmol/l (52-699 µmol/l).

Twenty-four out of 27 patients were thrombocytopenic (lowest platelet count <150 x 10⁹/l).

Thrombin generation by CAT®

When compared with the control phase, ETP was diminished by 16% (1154 nM/min, 67-1785 nM/min vs. 1385 nM/min, 670-1970 nM/min; p<0.001). Additionally, tt peak was prolonged (7.3 minutes, 4.8-14.9 minutes vs. 5.9 minutes, 4.3-9.8 minutes; p=0.012). Peak thrombin concentration was lowered (204 nM, 5.6-293 nM vs. 243 nM, 106-331 nM; p=0.008), and LT was prolonged (3.8 minutes, 2.1-7.7 minutes vs. 2.9 minutes, 2.0-4.1 minutes; p<0.001) in the acute phase. Accordingly, ETP correlated with peak thrombin
concentration ($r=0.833$, $p<0.001$; Fig. 1A). Tt peak associated with the LT ($r=0.78$, $p<0.001$; Fig. 1B). An inverse correlation was observed between peak and tt peak ($r= -0.54$, $p=0.004$).

**Associations of CAT® parameters with variables depicting clinical disease**

In the acute phase, ETP associated with the platelet count measured in the CAT® study day sample ($r=0.472$, $p=0.015$; Fig. 1C), and with the lowest platelet count measured during the hospital stay ($r=0.402$, $p=0.038$). Similarly, peak thrombin concentration associated with the platelet count of the CAT® study day ($r=0.554$, $p=0.003$), and with the lowest platelet count measured during the hospital stay ($r=0.462$, $p=0.015$). Prolonged LT and low ETP associated with increased fibrinogen level measured in the acute phase ($r=0.511$, $p=0.006$ and $r=0.386$, $p=0.047$, respectively; Fig. 1D). The fibrinogen level was acutely increased compared with the control phase (median 4.2, range 2.2-9.6 g/l and median 3.4 g/l, range 2.6-4.9 g/l, respectively; $p=0.005$).

D-dimer was increased in the acute phase (2.8 mg/l, 0.6-34 mg/l vs. 0.4 mg/l, 0.2-1.1 mg/l; $p<0.001$). D-dimer associated strongly with F1+2 ($r=0.843$, $p<0.001$). F1+2 was increased acutely (704 pmol/l, 284-1875 pmol/l vs. 263 pmol/l, 118-556 pmol/l; $p<0.001$). ETP did not associate with D-dimer and F1+2 ($r=-0.079$, $p=0.695$ and $r=-0.164$, $p=0.415$, respectively). There were no associations between peak and D-dimer and F1+2 ($r=-0.030$, $p=0.882$ and $r=-0.162$, $p=0.418$, respectively).

Clinical variables depicting the disease severity, i.e. maximum leukocyte count, maximum plasma creatinine, CRP level and length of hospital stay were not associated with CAT® parameters (data not shown).

Furthermore, reduced ETP was not associated with bleedings (data not shown).

**Discussion**

The primary aim of this study was to investigate whether thrombin generation (TG), measured using calibrated automated thrombography (CAT®) assay, is altered during acute Puumala virus-induced hemorrhagic fever with renal syndrome (PUUV-HFRS). In addition, we sought to elucidate the underlying coagulation mechanisms predisposing to bleeding and thrombosis. The main findings were reduced endogenous thrombin potential (ETP) and peak thrombin concentration suggesting diminished plasma potential for TG during acute PUUV-HFRS. Prolonged lag time (LT) indicating slower initiation of burst,
and extended time to reach the peak representing the velocity of TG, further support the finding of hypocoagulability. Decreased platelet count, one of the clinical characteristics of hantavirus infection, was found to associate with low ETP and low peak thrombin concentration. We did not find statistically significant associations between prothrombin fragments F1+2 (F1+2), D-dimer and CAT® parameters in this study population.

Previous studies imply enhanced TG in acute PUUV-HFRS on the basis of increased prothrombin fragments F1+2 generated during conversion of prothrombin to thrombin, and an increased level of fibrin degradation product D-dimer [8, 9, 11]. High levels of circulating F1+2 and D-dimer observed in this study confirm previous findings. In vivo TG parameters, F1+2 and D-dimer, strongly depend on the amount of tissue factor (TF) and thrombomodulin present on vascular endothelial cells [16, 18]. In vivo TG is also ongoing in the microparticles released during infection [19, 20]. Data suggests increased TF in endothelial cells in the acute phase of PUUV-HFRS [12]. On the other hand, the in vitro thrombin potential assessed by CAT® determines how thrombin can be generated by plasma containing a defined amount of TG trigger. Thus, it measures the haemostatic balance of plasma clotting factors and inhibitors independently of the procoagulant and inhibitory drivers released by the endothelium [18]. The difference between in vivo and in vitro TG is well reflected in consumption coagulopathy, a condition where indicators of ongoing coagulation are increased, but plasma potential of TG is decreased [21].

A previous study indicates slightly prolonged prothrombin time (PT), prolonged activated partial thromboplastin time (APTT), and shortened thrombin time (TT) during acute PUUV-HFRS [8, 18]. These in vitro coagulation tests cannot detect the in vivo contribution of endothelial cells and shear stress of blood flow on local clot formation and fibrinolysis. Traditional coagulation tests APTT and PT assess the time to the initiation of clot formation and thus they do not entirely reflect the hemostatic balance in acutely ill patients [22]. These plasma clotting assays are considered to reflect the LT phase of TG in CAT® assay [22].

Our observation of prolonged LT indicating slower initiation of TG is in line with the previous findings of the coagulation tests APTT and PT. As the data concerning in vitro coagulation tests is lacking in the current study population, the direct comparison with CAT® parameters is not possible.
Fibrinolysis is increased, as indicated by high D-dimer and endothelial cell tissue plasminogen activator (tPA) levels, during PUUV infection [8, 11-13]. The plasminogen activator inhibitor 1 (PAI-1) level is not altered in the acute phase [13]. Thrombomodulin-associated thrombin activates the thrombin activatable fibrinolysis inhibitor (TAFI), which downregulates fibrinolysis. It can be speculated that low ETP may contribute to decreased TAFI and thus increased fibrinolysis and bleeding tendency [23]. Diminished TG in PUUV-HFRS together with excessive fibrinolysis also resembles the data obtained in another hemorrhagic fever, dengue virus infection [24, 25]. In dengue fever the bleeding complications have been shown to associate with reduced thrombin formation along with thrombocytopenia and enhanced fibrinolysis [26]. We did not find an association between low ETP and bleedings, although mild bleedings were reported in one third of the patients.

We are aware of the relatively small sample size of the study. Yet, the associations were statistically significant, even if the number of clinical events remained minor. The levels of coagulation factors were not available and individual acquired or inherited factors affecting hemostasis could not be assessed. Two patients used aspirin, a platelet antagonist that inhibits platelet aggregation and thrombin formation. As TG was assessed in platelet poor plasma it is unlikely that aspirin, attached to the minor amount of residual platelets, could affect these CAT® results.

Ongoing in vivo coagulation may result in consumption of platelets and coagulation factors during acute PUUV-infection. Correlation of low platelet count with low ETP and low peak may imply thrombin activation and consumption of platelets, as thrombopoiesis is shown to be active during acute PUUV-HFRS [20]. Natural anticoagulants, protein C and protein S free antigen and antithrombin are also found to be decreased in the acute phase of PUUV-HFRS [8]. Increased TF expression on the endothelial cells and microparticle release might result in consumption of platelets and clotting factors resulting in lower ETP. All of these findings are supported by the previous reports [8, 11, 20].

In conclusion, in this study we found decreased in vitro TG measured by CAT® in acute PUUV infection. Together with thrombocytopenia, increased fibrinolysis and signs of enhanced TG in vivo this data suggests a mild to moderate consumption coagulopathy during acute PUUV-HFRS. The CAT® results of plasma...
hypocoagulobility support previous findings of impaired hemostasis during acute PUUV-HFRS. Larger future studies might further clarify the role of coagulation in the pathogenesis of HFRS.

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References


Figure 1. Scatter plots illustrating the correlation between endogenous thrombin potential and peak thrombin concentration (Fig. 1A), lag time and time to peak thrombin concentration (Fig. 1B), endogenous thrombin potential and simultaneous platelet count (Fig. 1C) and endogenous thrombin potential and plasma fibrinogen level (Fig. 1D) during acute PUUV infection.
Table 1. The clinical and laboratory findings during hospital care in 27 patients with acute Puumala hantavirus infection.

<table>
<thead>
<tr>
<th>Clinical or laboratory variable</th>
<th>Median</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days from the onset of illness(^a)</td>
<td>7</td>
<td>4-12</td>
</tr>
<tr>
<td>Length of hospital stay (days)</td>
<td>7</td>
<td>3-12</td>
</tr>
<tr>
<td>Body mass index (kg/m(^2))</td>
<td>26.6</td>
<td>22.3-36.8</td>
</tr>
<tr>
<td>Change in weight (kg)(^b)</td>
<td>3.8</td>
<td>0.5-11.3</td>
</tr>
<tr>
<td>Systolic BP min (mmHg)</td>
<td>108</td>
<td>80-135</td>
</tr>
<tr>
<td>Diastolic BP min (mmHg)</td>
<td>67</td>
<td>55-83</td>
</tr>
<tr>
<td>Creatinine max (µmol/l)</td>
<td>268</td>
<td>71-983</td>
</tr>
<tr>
<td>Leukocyte count max (x 10(^9)/l)</td>
<td>10.7</td>
<td>4-45</td>
</tr>
<tr>
<td>Hemoglobin max (g/l)</td>
<td>155</td>
<td>122-214</td>
</tr>
<tr>
<td>Hematocrit max</td>
<td>0.43</td>
<td>0.37-0.60</td>
</tr>
<tr>
<td>Platelet count min (x 10(^9)/l)</td>
<td>60</td>
<td>5-150</td>
</tr>
<tr>
<td>CRP max (mg/ml)</td>
<td>79</td>
<td>21-244</td>
</tr>
</tbody>
</table>

Abbreviations: min=minimum, max=maximum, BP=blood pressure.

Reference values: hematocrit 0.35-0.50 for men and 0.35-0.46 for women, platelet count 150-360, leukocyte count 3.4-8.2 x 10\(^9\)/l, CRP < 10 mg/ml, creatinine < 105 µmol/l for men and < 95 µmol/l for women.

\(^a\)The number of days of fever before the first study samples were obtained.

\(^b\)Reflects fluid accumulation in the oliguric phase of PUUV-HFRS.