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Elevated L-threonine is a biomarker for Lassa fever and Ebola

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ABSTRACT

Background: Lassa fever and Ebola are characterized by non-specific initial presentations that can progress to severe multisystem illnesses with high fatality rates.

Methods: Liquid Chromatography Mass Spectrometry metabolomics was used to identify and confirm metabolites disrupted in the blood of Lassa fever and Ebola patients.

Results: The amino acid L-threonine is elevated during Lassa fever and Ebola.

Conclusions: Metabolomics may provide tools to identify pathways that are differentially affected during viral hemorrhagic fevers and guide development of diagnostics to monitor and predict outcome.

Key words: Lassa fever, Ebola, viral hemorrhagic fevers, Liquid Chromatography Mass Spectrometry, L-threonine, metabolomics
BACKGROUND

Select features within the metabolome may serve as biomarkers for disease severity/progression/outcome and lend themselves to the design of prognostic methods for viral diseases, such as Lassa fever and Ebola. We have previously characterized endogenous small molecules with prognostic value originating from the blood of febrile patients triaged to the Lassa fever ward in Kenema, Sierra Leone [1]. Here, we corroborate biomarkers in samples from the most acutely ill Lassa fever patients and contrast select metabolites in patients with Ebola, another severe viral hemorrhagic fever (VHF).

Methods

Human Subjects

The Tulane University Institutional Review Board and the Sierra Leone Ethics and Scientific Research Committee approved this project. Patients were referred to the Kenema Government Hospital (KGH) Lassa Ward from the hospital’s general ward or from regional health centers on the basis of suspicion of Lassa fever. Patients who met the case definition of Lassa fever as defined by Khan et al. [2] were admitted and cared for by the ward’s trained staff. After the initial cases of Ebola were detected, patients were referred if they presented with an illness that met the World Health Organization case definition for Ebola. We obtained samples using the collection and processing protocols at Kenema Government Hospital under the emergency-response guidelines established by the Sierra Leone Ministry of Health and Sanitation. Diagnostic tests for the presence of Ebola virus (EBOV) were performed on site by means of quantitative reverse-transcriptase–polymerase chain reaction assays with the use of the SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase (Life Technologies).
Serum processing for metabolomics analyses

Small blood volumes (approximately 5 ml) for serum separation were collected from patients presenting to KGH with febrile illnesses that met preclinical criteria of suspected Lassa fever or Ebola. Patient samples received a coded designation and were collected in serum vacutainer tubes. Blood samples were allowed to coagulate for 20 minutes at room temperature. Serum was separated from coagulated blood by centrifugation (200 x g, 20 minutes at room temperature). For subjects for which there was excess serum not needed for clinical evaluations, aliquots of the serum fraction were stored in cryovials at -20°C prior to processing for metabolite analysis.

Serum metabolite analysis was performed as previously described [1]. Briefly, serum samples were depleted of protein by addition to one part sera (100 μL) of 4 parts ice-cold methanol (400 μL), the mixture was vortexed vigorously for 10 seconds, and incubated 1 hour at -20°C followed by centrifugation at 14,000 x g, 15 minutes, 4°C. The supernatant was collected and transferred to a new, sterile vial and dried under vacuum. The resultant small-molecule containing pellets were stored in desiccated, sealed containers and shipped to Tulane University where they were gamma-irradiated. Small molecule containing pellets were dissolved in a solution of 95:5 water:acetonitrile transferred to autosampler vials, and held at -20°C or 4°C immediately prior to analysis. All reagents utilized were high-pressure liquid chromatography (HPLC) grade.

Liquid Chromatography Mass Spectrometry

The Liquid Chromatography Mass Spectrometry (LCMS) method was performed as previously described with minor changes [1]. Briefly, detection of metabolites was performed via HPLC separation with ESI-MS (electrospray mass spectrometry) detection. HLPC was performed with
an aqueous normal-phase, hydrophilic interaction chromatography HPLC column: a Cogent Diamond Hydride Type-C column with 4 μm particles and dimensions of 150 mm length and 2.1 mm diameter or an Agilent Zorbax 300-SB-C18 column with 3.0 μm particles and dimensions of 150 mm length and 0.3 mm diameter was used with an Agilent 1290 HPLC system (Agilent Technologies, Santa Clara, CA). The column were maintained at 60°C with a flow rate of 900 μL/min. Chromatography was as follows: solvent consisted of H₂O with 0.1% (v/v) formic acid for channel “A” and acetonitrile with 0.1% formic acid for channel “B”. Following column equilibration at 98% B, the sample was injected via autosampler, and the column was flushed for 2.0 min to waste. From 2.0 min to 14.5 min, the gradient was linearly ramped from 98% to 65% B. From 14.5 min to 16.0 min, the gradient was ramped from 65% to 25% B. From 14.5 to 18.0 min the column was held at 25% B, and from 18.0 to 18.2 minutes the gradient was ramped from 25% to 98% B. From 18.2 to 20.0 minutes the column was re-equilibrated with 98% B. An Agilent 6538 Quad-Time of Flight with dual-electron spray ion source mass spectrometer was used for all analyses. Resolution was approximately 20,000 and accuracy was 1 ppm. Source parameters: drying gas 12 L/min, nebulizer 60 psi, capillary voltage 3500V, capillary exit 100V. Spectra were collected in positive mode from 50 to 1700 m/z at a rate of 1 Hz.

Molecular Standards

Authenticated standards of synthetic platelet-activating factor (PAF) C-16 (#878110) and lysoPAF C-16 (#878119) at a concentration of 5 mg/mL in chloroform were purchased from Avanti Polar Lipids (Birmingham, AL). L-Threonine (T8625) was purchased from Sigma-Aldrich. The molecules were diluted in 95:5 water:acetonitrile solution and analyzed with the identical method for metabolite detection.
Data Analysis and Visualization

Raw spectral data in .d format where uploaded to XCMS Online (Versions 2.3.0 or 2.2.3) and processed as pairwise comparisons using parameters optimized for data acquired with UPLC on an Agilent 6538 MS.

Statistics and Machine Learning

Statistical analyses were carried out using the R statistical software package or Graphpad Prism. Raw mass spectral intensity values and a unique identifier for specific spectral features were extrapolated from XCMS output and compiled into .csv file types.

RESULTS

Characteristics of a cohort of subjects presenting to Kenema Government Hospital with Lassa fever or Ebola

A panel of 50 serum samples from febrile patients triaged to a ward for the care of suspected Lassa fever patients was assembled to corroborate earlier observations of biomarkers of poor outcome in Lassa fever. Serum samples were drawn upon admittance and in all but two instances diagnostic tests were performed within 24 hours. Twenty-two subjects tested negative for Lassa virus (LASV) by all diagnostic tests and were classified as febrile non-Lassa. Subjects with a positive diagnosis for Lassa fever were subdivided into patients that were discharged (n=12) and those that succumbed to infection (n=16). Gender and age data were available for 48 of the 50 samples. Subjects were 54% female (26/48) with a mean age of 25.0 (years, range <1-60). There were 17
and 10 female and male LASV positive samples, respectively. Nine females and 6 males succumbed to Lassa fever with an average age of 21.5 (years, range <1-38) and an average time from symptom onset to death of 7.75 (days, 5-16). There were no significant differences between the distribution of LASV positive subjects or mortality between female and male subjects. A panel of serum samples derived from KGH collected during the 2015 EBOV outbreak in West Africa were also analyzed. Twelve febrile subjects tested negative for Lassa virus (LASV) and EBOV by all diagnostic tests and were classified as febrile non-Ebola. Twenty-nine subjects tested positive for EBOV at the time of hospitalization and were classified as Ebola positive.

L-threonine is elevated in VHF patients

A previously observed unknown molecular feature that is present in the sera of the most acutely ill Lassa fever patients has been identified as L-threonine (Fig. 1). Previous studies identified two features that were significantly elevated and detected as mass-to-charge ratio (m/z) 102.0537 and 119.0800 at identical retention times [rt = 15.95] [1]. These features have now been identified as the H+H2O− and NH4+H2O− adducts of L-threonine (Fig. 1A). In the present analysis the NH4+H2O− adduct of L-threonine is detected with m/z 119.0804 and significantly elevated in specimens from Lassa fever patients compared to febrile subjects without Lassa fever. The H+H2O− adduct of L-threonine was detected with m/z 102.0566 significantly elevated in Ebola specimens or endemic non-Ebola samples (Fig. 1B). L-threonine was not elevated in the blood of convalescent Ebola patients. These results show that elevated L-threonine may be a common marker of acute VHF as patients positive for both EBOV and LASV infection have significant elevation of this compound in the blood across spatially and temporally distinct disease outbreaks.
Platelet-activating factor and platelet-activating factor like-lipids are decreased in subjects with fatal Lassa fever

Platelet activity is depressed during Lassa fever, particularly in terminal patients [3, 4]. Twenty-four PAF or PAF-like molecules were putatively identified and expressed at variable levels in the serum of febrile patients presenting to KGH (Fig. 2). The cluster analysis indicated that nearly all PAFs or PAF-like molecules were present in lower amounts in the serum of patients with fatal Lassa fever than in patients that survived the acute infection (nonfatal Lassa fever). Non-Lassa febrile illness patients had the highest overall levels of PAF or PAF-like molecules. We also positively confirmed the identity of PAF and lysoPAF with LCMS versus authenticated standards in the serum of patients with Lassa fever. PAF and molecules with PAF moiety are reduced in severely ill and terminal Lassa fever patients. These lipids were not observed to be similarly dysregulated in a limited cohort of serum samples of Ebola positive patients compared to convalescent Ebola and febrile non-Ebola patients.

DISCUSSION

A previously unknown molecule elevated in fatal, acute, and post-Lassa acute febrile patients has been identified as L-threonine. The serum of Ebola patients also has elevated levels of L-threonine. Results showing elevation of L-threonine in the blood of Lassa fever and Ebola patients confirms and extend results previously published by Eisfeld and coworkers [5]. These investigators demonstrated that levels of serum L-threonine, as well as a vitamin D binding protein, perfectly stratified Ebola patients by outcome, providing better predictive ability than viral load. The amniotic fluid of human cytomegalovirus infected (HCMV) women shows elevated levels of L-threonine compared to non-HCMV infected women [6]. No significance difference in vertical
transmission of HCMV based on elevated L-threonine was observed. Glycine, serine, and threonine metabolic pathways were found to be altered in chikungunya or dengue patients [7]. Further studies are required to determine whether elevated serum L-threonine represents a general marker of VHF or other severe viral infections. We also confirmed that PAF and molecules with PAF moiety are reduced in the blood of patients with fatal Lassa fever. Similar changes were not observed in the blood of Ebola patients.

CONCLUSIONS
Metabolomics may provide tools to identify pathways that are differentially affected during VHFs. For patients suffering from VHF it would also be advantageous to have a measure of disease progression/severity, such as L-threonine levels, to predict outcomes several days prior to death. Coupling the detection and changes of these and other analytes with appropriate rapid diagnostics through disease progression may serve as a mechanism to monitor and predict outcome, ensuring scarce resources are allocated where needed most.

List of Abbreviations
viral hemorrhagic fever: VHF; Kenema Government Hospital: KGH; Ebola virus: EBOV; Lassa virus: LASV; mass-to-charge ratio: m/z; high-pressure liquid chromatography: HPLC; Liquid Chromatography Mass Spectrometry: LCMS; aqueous normal-phase, human cytomegalovirus infected: HCMV; Viral Hemorrhagic Fever Consortium: VHFC.
Ethics approval and consent to participate

The Tulane University Institutional Review Board (191330) and the Sierra Leone Ethics and Scientific Research Committee (070716) approved this project.

Consent to publication

Not Applicable.

Availability of data and material

Data has been deposited in the XCMS Public archive (https://xcmsonline.scripps.edu/landing_page.php?pgcontent=mainPage) under the identifier: 1181466.

Competing interests

JSS, LMB, RFG and DSG are members of the VHFC (www.vhfc.org). The VHFC is a partnership of academic and industry scientists who are developing diagnostic tests, therapeutic agents, and vaccines for Lassa fever, Ebola, and other severe diseases. Tulane University and its various academic and industry partners have filed US and foreign patent applications on behalf of the consortium for several of these technologies. Technical information may also be kept as trade secrets. If commercial products are developed, consortium members may receive royalties or profits. This does not alter our adherence to all policies of the NIH and Virology Journal on sharing data and materials. Financial and non-financial competing interests that the editors consider relevant to the content of the manuscript have been disclosed. RFG and LMB are co-founders of Zalgen. LMB is a Zalgen employee. All other authors declare no competing interests.
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5 Authors’ Contributions
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7 J.S.S., L.M.B., R.F.G., D.S.G.; Writing – Original Draft, T.V.G; Writing – T.V.G. L.M.B, R.F.G.
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Figure 1. Signal intensity of serum threonine from viral hemorrhagic fever patients. Signal intensity of serum threonine values are presented as mean and SEM. Panel A: LASV-infected patients with fatal or non-fatal outcome and febrile control subjects. Panel B: EBOV-infected patients and febrile control subjects. Significance levels (p values from one way ANOVA) are ***p < 0.0005 and ****p < 0.0001.
Figure 2. Cluster analysis of selected serum lipids in subjects with different Lassa virus serostatus and outcomes. The heat map represents levels of 24 putatively identified platelet activating factor (PAF) or PAF-like molecules.
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