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Guidance for Industry

**Use of Serological Tests to Reduce the Risk of Transmission of
Trypanosoma cruzi Infection in Whole Blood and Blood Components
for Transfusion and Human Cells, Tissues, and Cellular and
Tissue-Based Products (HCT/Ps)**

This draft guidance, when finalized, will represent the Food and Drug Administration's (FDA's) current thinking on this topic. It does not create or confer any rights for or on any person and does not operate to bind FDA or the public. You can use an alternative approach if the approach satisfies the requirements of the applicable statutes and regulations. If you want to discuss an alternative approach, contact the appropriate FDA staff. If you cannot identify the appropriate FDA staff, call the appropriate number listed on the title page of this guidance.

I. INTRODUCTION

We, FDA, are notifying you, establishments that manufacture Whole Blood and blood components intended for use in transfusion, and establishments that make eligibility determinations for donors of HCT/Ps, about FDA approval of a Biologics License Application (BLA) for an enzyme-linked immunosorbent assay (ELISA) test system for the detection of antibodies to *Trypanosoma cruzi* (*T. cruzi*). This test is intended for use as a donor screening test to reduce the risk of transmission of *T. cruzi* infection by detecting antibodies to *T. cruzi* in plasma and serum samples from individual human donors, including donors of Whole Blood and blood components intended for use in transfusion, and HCT/P donors (living and cadaveric (non-heart beating)). This guidance document does not apply to the collection of Source Plasma.

In addition, we are providing you with recommendations for unit and donor management, labeling of Whole Blood and blood components, and procedures for reporting implementation of a licensed *T. cruzi* test at your facility or at your contract testing laboratory, as required for blood establishments under Title 21 Code of Federal Regulations 601.12 (21 CFR 601.12). For establishments that make donor eligibility determinations for HCT/P donors, we are notifying you that we have determined *T. cruzi* to be a relevant communicable disease agent under 21 CFR 1271.3(r)(2), and are providing you with recommendations for testing and screening donors for antibodies to *T. cruzi*.

The recommendations made in this guidance with respect to HCT/Ps are in addition to recommendations made in the document entitled "Guidance for Industry: Eligibility Determination for Donors of Human Cells, Tissues, and Cellular and Tissue-Based Products (HCT/Ps)," dated August 2007 (Ref. 1).

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We recommend that you implement the recommendations provided in this guidance within one year after a final guidance is issued.

FDA's guidance documents, including this guidance, do not establish legally enforceable responsibilities. Instead, guidances describe FDA's current thinking on a topic and should be viewed only as recommendations, unless specific regulatory or statutory requirements are cited. The use of the word *should* in FDA's guidances means that something is suggested or recommended, but not required.

II. BACKGROUND

Chagas disease is caused by the protozoan parasite, *T. cruzi*. The disease is found primarily in Mexico and Central and South America; the pathogenic agent has rarely been reported to cause human infection in the United States (U.S.) by natural vector transmission (Ref. 2). Natural infections are transmitted mainly when the feces of certain blood sucking insects (triatomine bugs, commonly referred to as kissing or chinch bugs) that harbor the infection are rubbed into a bug bite, other wound, or directly into the eyes or mucous membranes. Other primary forms of transmission include congenital (mother to unborn infant), organ transplantation, and blood transfusion. Current estimates are that at least 11 million persons in Mexico and Central and South America carry the parasite chronically and could present a potential source of infection should they become donors. The presence of the pathogenic agent in U.S. and Canadian donors is increasing due to immigration of infected individuals from endemic areas. Some experts estimate that there may be as many as 100,000 persons unknowingly infected with *T. cruzi*, who reside in the U.S. and Canada.

Vector-borne infections are mostly mild in the acute phase and then persist throughout life, usually without symptoms. Acute infection in patients with compromised immune systems, for example, from cancer therapy or organ transplantation, can be very serious and sometimes fatal. Treatment options are limited, but are most effective early in the infection. The lifetime risk of severe cardiac complications (cardiomegaly, heart failure and arrhythmias) or intestinal disorders (megacolon, megaesophagus) in infected individuals averages about 30% (range of 10 to 40% depending on a variety of factors) and may occur many years after the initial infection. During the acute phase of vector-borne Chagas disease, parasites are found in skin lesions at the site of transmission. The parasites are then spread through the bloodstream to various tissues, particularly skeletal muscle (Ref. 3). During the chronic stage of Chagas disease, most persons who harbor the parasite are asymptomatic and unaware of their infection. During this phase, parasites have been demonstrated in muscle (especially cardiac muscle), nerves, and digestive tract, but there has been very little investigation of tissue distribution during that phase (Refs. 3 through 10).

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A. Donor Screening Tests for Chagas Disease in the United States

At the September 1989 Blood Products Advisory Committee (BPAC) meeting, the committee recommended testing donors of Whole Blood and blood components for Chagas disease when a suitable test became available. In a 1995 BPAC meeting, the committee considered whether the performance characteristics of the two FDA-approved tests then available for diagnosis of Chagas disease would be suitable for blood donor screening. The committee concluded that the tests discussed were not suitable for blood donor screening. Furthermore, the committee sought clarification of the criteria that FDA would use to license a Chagas test for donor screening. At the September 2002 meeting of BPAC, FDA presented its current considerations on the regulatory pathway and standards for licensing a donor screening test for Chagas disease and encouraged manufacturers to develop tests based on those considerations (Ref. 11).

In December 2006, FDA granted a license to one manufacturer of an ELISA test system for the detection of antibodies to *T. cruzi* in individual living blood and HCT/P donors. Since the end of January 2007, a number of blood centers representing a large proportion of U.S. blood collections have been testing donors using this licensed assay. In February 2009, FDA licensed this ELISA test system for the detection of antibodies to *T. cruzi* in cadaveric (non-heart beating) HCT/P donors.

Blood donor testing by an ELISA test system identifies donors that are repeatedly reactive for antibodies to *T. cruzi*. The presence of antibodies to *T. cruzi* is strong evidence that a donor is infected with this parasite. Most donors that are repeatedly reactive by an ELISA test system for antibodies to *T. cruzi* have chronic, asymptomatic infections acquired years earlier during residence in areas endemic for *T. cruzi*. Therefore, prior donations from a donor who is repeatedly reactive on an ELISA test system were likely to harbor *T. cruzi* parasites.

At the April 2007 BPAC meeting, FDA requested comments on scientific issues related to the implementation of blood donor testing for infection with *T. cruzi* (Ref. 12). Issues discussed by the committee included the need for additional data on the incidence and risk of transmission of *T. cruzi* by transfusion, the severity of Chagas disease, the performance of the antibody test, and, the lack of a licensed supplemental test for confirmatory testing.

The committee also commented on the design of research studies to validate a strategy for selective testing of repeat blood donors. The committee noted that a period of universal testing of all blood donors would generate critical data on the prevalence of *T. cruzi* infections in donors and that donor questions for selective donor screening needed validation.

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B. Risk of *T. cruzi* Infection from Transfusion of Whole Blood and Blood Components

Blood donations from individuals from endemic areas are the primary source of risk for *T. cruzi* infection from transfusion. Studies in the mid-1990s (Ref. 1) estimated that the rate of seropositive blood donors in the U.S. ranged from 1 in 5400 to 1 in 25,000, depending on where the studies were conducted. However, more recent studies suggest that these rates have increased in the areas where donor testing has been performed over a period of time. For example, a rate of 1 in 2000 was found recently in the Los Angeles metropolitan area (Ref. 14). Transfusion transmission in endemic areas has been a major public health concern, and many countries considered endemic for *T. cruzi* infection screen blood donors for the presence of antibody. Therefore, in response to changes in donor demographics, we are now recommending blood donor testing in the U.S.

In the U.S. and Canada, only seven cases of transfusion-transmitted *T. cruzi* infections (Refs. 15 through 19) and five cases of infection from organ transplantation (Refs. 20 and 21) have been documented. However, transmission in immunocompetent patients is not likely to be apparent, and in many cases, even if symptoms appear, infection may not be recognized (Ref. 22).

Studies in blood centers which question donors about birth and/or residence in a *T. cruzi*-endemic country have shown such questions to be incompletely effective at identifying the seropositive donors. Studies also have looked at the rate of transfusion transmission from *T. cruzi* antibody-positive individuals. Published lookback studies in the U.S. and in Mexico of 22 transfusion recipients of seropositive donations, identified five of these recipients (22.7%) who later tested positive for antibodies suggesting transfusion transmission of *T. cruzi* (Refs. 18, 23 and 24). This transmission rate of 22.7% is consistent with the literature from Latin America on rates of blood-borne transmission from seropositive donors in Mexico and Central and South America (Ref. 25). However, we are aware that lookback studies conducted using the licensed ELISA test indicate that the risk of *T. cruzi* by transfusion of a seropositive unit in the U.S. may be much lower risk than previously thought. We note that these studies have confirmed the demographic characteristics of the typical seropositive donor as described in the first two paragraphs of section II. However, the data also suggest that there are seropositive individuals who acquired their infections within the U.S. (Ref. 26). Despite this new data, the rate of transfusion transmission of *T. cruzi* in the U.S. continues to be uncertain because of the limited number of studies conducted to date and the rate of transfusion transmission remains under investigation.

C. Risk of *T. cruzi* Infection to Recipients of Donated HCT/Pls

Based on the risk of transmission, severity of effect, and availability of appropriate screening measures and/or tests, we have determined *T. cruzi*, the agent for Chagas disease, to be a relevant communicable disease agent or disease under 21 CFR 1271.3(r)(2). This determination was based on the following information.

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1. Risk of Transmission

There is a risk of transmission of *T. cruzi* by HCT/Ps and there has been sufficient incidence and/or prevalence to affect the potential donor population.

Recognizing the risk of transmission from donated HCT/Ps, countries endemic for *T. cruzi* infection have instituted various practices to minimize transmission through transfusion or transplantation including screening donors for the presence of *T. cruzi* antibodies. Further, when human leukocyte antigen-matched bone marrow is obtained from an infected individual, the donor receives anti-parasitic treatment before the bone marrow is taken for transplantation. The World Health Organization recommends that:

- a heart from an infected donor not be transplanted;
- a liver from an infected donor only be transplanted to recipients already positive for Chagas disease, except in emergency cases; and
- when other organs are transplanted from a Chagas-positive donor, the recipient should receive prophylactic treatment for Chagas disease (Ref. 3).

Published data regarding the transmissibility of *T. cruzi* indicate that vertical transmission (congenitally from mother to infant), oral transmission (through breast milk or contaminated food) and conjunctival transmission (from contact with contaminated hands) have occurred (Ref. 3). In animal studies, *T. cruzi* has been shown to infect multiple tissues, including skeletal muscle, heart, bladder, peripheral nerve, liver, spleen, adrenal gland, brain, adipose tissue, ocular tissue, osteoblasts, chondroblasts, macrophages, and fibroblasts (Refs. 27 through 30). Human placental cells also have been experimentally infected with *T. cruzi* (Ref. 31). As noted previously in this section, *T. cruzi* has been transmitted via blood transfusions and organ transplantation (Refs. 20 through 22, and 32).

At the BPAC meeting of April 26, 2007, the committee noted that, though some HCT/Ps are processed in a manner that might inactivate *T. cruzi* in HCT/Ps from seropositive donors, current data are insufficient to identify specific effective processing methods that consistently render HCT/Ps free of *T. cruzi*. The committee concluded that, absent such data, it would be prudent to test HCT/P donors to decrease the risk of transmitting infection with *T. cruzi* (Ref. 12).

Information about prevalence of *T. cruzi* in the U.S. is provided in section II.B. of this document.

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2. Severity of Effect

T. cruzi infections can be fatal or life-threatening, result in permanent impairment of a body function or permanent damage to a body structure, and/or necessitate medical or surgical intervention to preclude permanent impairment of a body function or permanent damage to a body structure.

3. Availability of Appropriate Screening and/or Testing Measures

Appropriate screening measures have been developed for *T. cruzi*, such as the medical history interview. (Screening measures for *T. cruzi* are discussed in section IV.A. of this document.)

A donor screening test for *T. cruzi* has been licensed and labeled for use in testing blood specimens from living and cadaveric donors of HCT/Ps (see section IV.B. of this document). You must use a donor screening test for *T. cruzi* that is specifically labeled for cadaveric specimens instead of a more generally labeled donor screening test when applicable and when available (21 CFR 1271.80(c)). Current FDA-licensed, cleared or approved donor screening tests for use in testing HCT/P donors are listed at <http://www.fda.gov/cber/tissue/prod.htm>.

III. RECOMMENDATIONS FOR DONORS OF WHOLE BLOOD AND BLOOD COMPONENTS INTENDED FOR USE IN TRANSFUSION

A. Blood Donor Testing and Management

1. Donor Testing

We recommend testing of all donations of allogeneic units of blood using a licensed test for antibodies to *T. cruzi*. You must follow the regulations under 21 CFR 610.40(d) for determining when autologous donations must be tested.

2. Donor Deferral

We recommend that all donors who are repeatedly reactive on a licensed test for *T. cruzi* antibody or who have a history of Chagas disease be indefinitely deferred and notified of their deferral.

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3. Confirmatory Testing and Donor Reentry

At this time, there is no FDA licensed supplemental test for antibodies to *T. cruzi* that can be used for confirmation of true positive screening test results. FDA is not recommending reentry criteria for blood donors deferred indefinitely on the basis of a repeatedly reactive screening test for antibodies to *T. cruzi* due to the absence of a licensed supplemental test for antibodies to *T. cruzi*.

4. Donor Counseling and Physician Referral

We recommend that donors who are repeatedly reactive using a licensed test for antibodies to *T. cruzi* be informed about the likelihood and medical significance of infection with *T. cruzi*. Additional medical diagnostic testing may provide information useful in donor counseling.

All repeatedly reactive donors should be referred to a physician specialist. It also may be useful to refer them to their state and local health departments or to other appropriate community resources.

5. Further Testing of Repeatedly Reactive Donors for Cross-Reacting Diseases

Because the licensed test has demonstrated some reactivity in donors infected with pathogens other than *T. cruzi*, we recommend that medical follow up be considered for donors who are repeatedly reactive by the licensed test for antibodies to *T. cruzi* but who have no apparent basis for exposure to *T. cruzi* or who have negative results on more specific medical diagnostic tests. For example, testing for leishmaniasis may be appropriate in persons with geographic risk for exposure to *Leishmania* parasites and who appear to have a falsely reactive screening test for antibodies to *T. cruzi*.

B. Product Management

1. Index Donations

We recommend that blood components from repeatedly reactive index donations be quarantined and destroyed or used for research. Components determined to be unsuitable for transfusion must be prominently labeled: "NOT FOR TRANSFUSION," and the label must state the reason the unit is considered unsuitable (e.g., the component is positive for *T. cruzi* (21 CFR 606.121(f)).

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2. Lookback (Product Retrieval and Recipient Notification)

Within 3 calendar days after a donor tests repeatedly reactive by a licensed test for *T. cruzi* antibody, you should:

- identify all in-date blood and blood components previously donated by such a donor, going back either 10 years (or indefinitely where electronic records are available), or else 12 months prior to the most recent time that this donor tested negative with a licensed test for *T. cruzi* antibody, whichever is the lesser period (the lookback period);
- quarantine all previously collected in-date blood and blood components held at your establishment; and
- notify consignees of all previously collected in-date blood and blood components to quarantine and return the blood components to you or to destroy them.

In addition, when you identify a donor who is repeatedly reactive by a licensed test for *T. cruzi* antibodies and for whom there is additional information indicating risk of *T. cruzi* infection, such as geographical risk for exposure in an endemic area, or medical diagnostic testing of the donor, we recommend that you:

- notify consignees of all previously distributed blood and blood components collected during the lookback period; and
- if blood or blood components were transfused, encourage consignees to notify the recipient's physician of record of a possible increased risk of *T. cruzi* infection.

We recommend that when there is additional information indicating risk of *T. cruzi* infection you make such notifications within 12 weeks of obtaining the repeatedly reactive test result.

There currently is no licensed *T. cruzi* supplemental test. When such a test is available, a positive test result will provide additional information indicating risk of *T. cruzi* infection.

Retrospective Review of Records

If you are a blood establishment that implemented screening with a licensed test for antibodies to *T. cruzi* prior to the effective date of this guidance, you may wish to perform a retrospective review of records to identify donors:

- with repeatedly reactive test results by a licensed test for *T. cruzi* antibodies; and
- for whom there is additional information indicating risk of *T. cruzi* infection, such as geographical risk for exposure in an endemic area, or medical diagnostic testing of the donor. There currently is no licensed *T.*

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If a donor is identified at risk of infection during the retrospective review, you may want to consider performing all the lookback actions described above.

3. Autologous Donations

Although autologous use of blood does not increase a patient's/donor's risk of illness from a pre-existing infection, FDA regulations under 21 CFR 610.40(d) and (e) require testing of autologous blood donors under certain circumstances to prevent inadvertent allogeneic exposures to unsuitable units.

a. We recommend that blood components from autologous donors that are repeatedly reactive by a licensed test for *T. cruzi* antibody be released for autologous use only with approval of the autologous donor's referring physician. Establishments should provide the results of additional testing for antibodies to *T. cruzi*, as available to the autologous donor's referring physician.

b. Each autologous donation must be labeled as required under 21 CFR 610.40(d)(4), as appropriate. Given the seriousness of *T. cruzi* infections, autologous donations that are repeatedly reactive by a licensed test for *T. cruzi* antibody must bear a biohazard label as required under 21 CFR 610.40(d)(4).

4. Circular of Information

Consistent with other donor screening tests, the instruction circular, also known as the "Circular of Information" must be updated to state that a licensed test for antibodies to *T. cruzi* was used to screen donors and that the results of testing were negative (21 CFR 606.122(h)).

5. Biological Product Deviation Report and Fatality Report

Under 21 CFR 606.171, licensed manufacturers, unlicensed registered blood establishments, and transfusion services must report any event and information associated with the manufacturing, if the event either represents a deviation from current good manufacturing practice, applicable regulations, applicable standards, or established specifications that may affect the safety, purity, or potency of the product; or represents an unexpected or unforeseeable event that may affect the safety, purity, or potency of the product, and it occurs in your facility or another facility under contract with you and involves distributed blood or blood components. For additional information regarding reporting, you may refer to

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FDA guidance, “Guidance for Industry: Biological Product Deviation Reporting for Blood and Plasma Establishments,” dated October 2006 (Ref. 33). Also, when a complication of blood collection or transfusion (e.g., involving *T. cruzi*) is confirmed to be fatal, you must notify FDA in accordance with 21 CFR 606.170(b).

C. Reporting the Test Implementation

1. If you are a licensed blood establishment and you begin using a licensed serological test for the detection of antibodies to *T. cruzi* according to the manufacturer’s product insert at your facility, then you must notify us of the testing change in your Annual Report (AR), in accordance with 21 CFR 601.12(d). If you already have an approved supplement to your BLA to use a contract laboratory to perform infectious disease testing of blood products, and the contract laboratory will now perform a serological test for antibodies to *T. cruzi*, you must report this change in your AR (21 CFR 601.12(d)).
2. If you are a licensed blood establishment and you use a new contract laboratory to perform a serological test for antibodies to *T. cruzi* (and the laboratory already performs infectious disease testing for blood products), then you must report this change by submission of a “Changes Being Effected” supplement, in accordance with 21 CFR 601.12(c)(1) and (c)(5). If your contract laboratory has not previously performed infectious disease testing for blood products, then you must report this change as a major change in a prior approval supplement, in accordance with 21 CFR 601.12(b).

IV. RECOMMENDATIONS FOR DONORS OF HCT/PS

A. Donor Screening—Risk Factors or Conditions

Under 21 CFR 1271.75(d), you must determine to be ineligible any potential donor who is identified as having a risk factor for or clinical evidence of relevant communicable disease agents or diseases. Ineligible potential donors include those who exhibit one or more of the following conditions or behaviors.

- Persons who have had a medical diagnosis of *T. cruzi* infection based on symptoms and/or laboratory results.
- Persons who have tested positive or reactive for *T. cruzi* antibodies using an FDA-licensed or investigational *T. cruzi* donor screening test (Ref. 1).

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B. Donor Testing

1. You must test blood specimens from all HCT/P donors for antibodies to *T. cruzi* using an FDA-licensed donor screening test (21 CFR 1271.80(c)).
2. Any HCT/P donor whose specimen tests negative (or non-reactive) for antibodies to *T. cruzi* may be considered to be negative (or non-reactive) for purposes of making a donor eligibility determination.
3. Any HCT/P donor whose specimen tests positive (or reactive) for antibodies to *T. cruzi* is ineligible to be a donor (21 CFR 1271.80(d)(1)).

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医薬品 研究報告 調査報告書

識別番号・報告回数		報告日	第一報入手日 2009. 6. 5	新医薬品等の区分 該当なし	総合機構処理欄
一般的名称	人血清アルブミン	研究報告の公表状況	Briese T, Paweska JT, McMullan LK, Hutchison SK, Street C, Palacios G, Khristova ML, Weyer J, Swanepoel R, Egholm M, Nichol ST, Lipkin WI. PLoS Pathog. 2009 May;5(5):e1000455. Epub 2009 May 29.	公表国	
販売名(企業名)	赤十字アルブミン20(日本赤十字社) 赤十字アルブミン25(日本赤十字社) 赤十字アルブミン20%静注4g/20mL(日本赤十字社) 赤十字アルブミン20%静注10g/50mL(日本赤十字社) 赤十字アルブミン25%静注12.5g/50mL(日本赤十字社)		米国		
研究報告の概要	○アフリカ南部で発見された新規出血熱関連アレナウイルス、Lujoウイルスの遺伝子検出と特徴 Lujoウイルス(LUJV)はアレナウイルス科の新たなウイルスで、過去30年間で初めて発見された出血熱関連旧世界アレナウイルスである。LUJVは南アフリカにおけるヒト疾患のアウトブレイク中に分離され、院内感染とこれまでにない高い死亡率(4/5例、80%)が特徴である。アウトブレイクの死亡患者から採取した血漿及び組織由来のRNA抽出物の無作為パイロシーケンスにより、検体受領から72時間以内で同定と詳細な系統発生的特徴の分析ができた。LUJVの全ゲノム分析では、かなり昔に旧世界アレナウイルスから分岐して独特の配列を持っていることが判明した。ウイルスのG1糖タンパク質シーケンスは、他の旧世界/新世界アレナウイルスとは大きく異なって両者のほぼ中間に位置し、特徴的なレセプター親和性を持っていた。LUJVは系統的に独立した新しい高病原性アレナウイルスである。			使用上の注意記載状況・ その他参考事項等	
				赤十字アルブミン20 赤十字アルブミン25 赤十字アルブミン20%静注 4g/20mL 赤十字アルブミン20%静注 10g/50mL 赤十字アルブミン25%静注 12.5g/50mL 血液を原料とすることによる 感染症伝播等	
報告企業の意見		今後の対応			
南アフリカにおいて、系統的に独立した新しい高病原性アレナウイルスのLujoウイルスが検出されたとの報告である。アレナウイルスは脂質膜を持つ比較的大型のRNAウイルスである。これまで、本剤によるアレナウイルス感染の報告はない。本剤の製造工程には、平成11年8月30日付医薬発第1047号に沿ったウイルス・プロセスバリデーションによって検証された2つの異なるウイルス除去・不活化工程が含まれていることから、本剤の安全性は確保されていると考える。		念のため今後も情報収集に努める。なお、日本赤十字社では帰国(入国)後4週間は献血不適とし、輸入感染症の防止に努めている。			

Genetic Detection and Characterization of Lujo Virus, a New Hemorrhagic Fever–Associated Arenavirus from Southern Africa

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Abstract

Lujo virus (LUJV), a new member of the family *Arenaviridae* and the first hemorrhagic fever-associated arenavirus from the Old World discovered in three decades, was isolated in South Africa during an outbreak of human disease characterized by nosocomial transmission and an unprecedented high case fatality rate of 80% (4/5 cases). Unbiased pyrosequencing of RNA extracts from serum and tissues of outbreak victims enabled identification and detailed phylogenetic characterization within 72 hours of sample receipt. Full genome analyses of LUJV showed it to be unique and branching off the ancestral node of the Old World arenaviruses. The virus G1 glycoprotein sequence was highly diverse and almost equidistant from that of other Old World and New World arenaviruses, consistent with a potential distinctive receptor tropism. LUJV is a novel, genetically distinct, highly pathogenic arenavirus.

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Introduction

Members of the genus *Arenavirus*, comprising currently 22 recognized species (<http://www.ictvonline.org/virusTaxonomy.asp?version=2008>), are divided into two complexes based on serologic, genetic, and geographic relationships [1,2]: the New World (NW) or Tacaribe complex, and the Old World (OW) or Lassa-Lymphocytic choriomeningitis complex that includes the ubiquitous arenavirus type-species *Lymphocytic choriomeningitis virus* (LCMV; [3]). The RNA genome of arenaviruses is bi-segmented, comprising a large (L) and a small (S) segment that each codes for two proteins in ambisense coding strategy [4,5]. Despite this coding strategy, the *Arenaviridae* are classified together with the families *Orthomyxoviridae* and *Bunyaviridae* as segmented single-strand, negative sense RNA viruses.

The South American hemorrhagic fever viruses Junin (JUNV; [6,7]), Machupo (MACV; [8]), Guanarito (GTOV; [9]) and Sabia virus (SABV, [10]), and the African Lassa virus (LASV [11]), are restricted to biosafety level 4 (BSL-4) containment due to their associated aerosol infectivity and rapid onset of severe disease. With the possible exception of NW Tacaribe virus (TCRV; [12]), which has been isolated from bats (*Artibeus* spp.), individual arenavirus species are commonly transmitted by specific rodent species wherein the capacity for persistent infection without overt

disease suggests long evolutionary adaptation between the agent and its host [1,13–16]. Whereas NW arenaviruses are associated with rodents in the *Sigmodontinae* subfamily of the family *Cricetidae*, OW arenaviruses are associated with rodents in the *Murinae* subfamily of the family *Muridae*.

Humans are most frequently infected through contact with infected rodent excreta, commonly via inhalation of dust or aerosolized virus-containing materials, or ingestion of contaminated foods [13]; however, transmission may also occur by inoculation with infected body fluids and tissue transplantation [17–19]. LCMV, which is spread by the ubiquitous *Mus musculus* as host species and hence found world-wide, causes symptoms in humans that range from asymptomatic infection or mild febrile illness to meningitis and encephalitis [13]. LCMV infection is only rarely fatal in immunocompetent adults; however, infection during pregnancy bears serious risks for mother and child and frequently results in congenital abnormalities. The African LASV, which has its reservoir in rodent species of the *Mastomys* genus, causes an estimated 100,000–500,000 human infections per year in West African countries (Figure 1). Although Lassa fever is typically sub-clinical or associated with mild febrile illness, up to 20% of cases may have severe systemic disease culminating in fatal outcome [20,21]. Three other African arenaviruses are not known to cause human disease: Ippy virus (IPPYV; [22,23]), isolated from

Author Summary

In September and October 2008, five cases of undiagnosed hemorrhagic fever, four of them fatal, were recognized in South Africa after air transfer of a critically ill index case from Zambia. Serum and tissue samples from victims were subjected to unbiased pyrosequencing, yielding within 72 hours of sample receipt, multiple discrete sequence fragments that represented approximately 50% of a prototypic arenavirus genome. Thereafter, full genome sequence was generated by PCR amplification of intervening fragments using specific primers complementary to sequence obtained by pyrosequencing and a universal primer targeting the conserved arenaviral termini. Phylogenetic analyses confirmed the presence of a new member of the family *Arenaviridae*, provisionally named Lujo virus (LUJV) in recognition of its geographic origin (Lusaka, Zambia, and Johannesburg, South Africa). Our findings enable the development of specific reagents to further investigate the reservoir, geographic distribution, and unusual pathogenicity of LUJV, and confirm the utility of unbiased high throughput pyrosequencing for pathogen discovery and public health.

Arvicantis spp. and Mobala virus (MOBV; [24]) isolated from *Praomys* spp. in the Central African Republic (CAR); and Mopeia virus (MOPV) that like LASV is associated with members of the genus *Mastomys*, and was reported from Mozambique [25] and Zimbabwe [26], although antibody studies suggest that MOPV and LASV may also circulate in CAR [27] where the geographies of these viruses appear to overlap (Figure 1). Up to present, there have been no published reports of severe human disease associated with arenaviruses isolated from southern Africa.

In September 2008 an outbreak of unexplained hemorrhagic fever was reported in South Africa [28]. The index patient was airlifted in critical condition from Zambia on September 12 to a clinic in Sandton, South Africa, after infection from an unidentified source. Secondary infections were recognized in a paramedic (case 2) who attended the index case during air transfer from Zambia, in a nurse (case 3) who attended the index case in the intensive care unit in South Africa, and in a member of the hospital staff (case 4) who cleaned the room after the index case died on September 14. One case of tertiary infection was recorded in a nurse (case 5) who attended case 2 after his transfer from Zambia to Sandton on September 26, one day before barrier nursing was implemented. The course of disease in cases 1 through 4 was fatal; case 5 received ribavirin treatment and recovered. A detailed description of clinical and epidemiologic data, as well as immunohistological and PCR analyses that indicated the presence of an arenavirus, are reported in a parallel communication (Paweska et al., *Emerg. Inf. Dis.*, submitted). Here we report detailed genetic analysis of this novel arenavirus.

Results/Discussion

Rapid identification of a novel pathogen through unbiased pyrosequencing

RNA extracts from two post-mortem liver biopsies (cases 2 and 3) and one serum sample (case 2) were independently submitted for unbiased high-throughput pyrosequencing. The libraries yielded between 87,500 and 106,500 sequence reads. Alignment of unique singleton and assembled contiguous sequences to the GenBank database (<http://www.ncbi.nlm.nih.gov/Genbank>) using the Basic Local Alignment Search Tool (blastn and blastx;

[29]) indicated coverage of approximately 5.6 kilobases (kb) of sequence distributed along arenavirus genome scaffolds: 2 kb of S segment sequence in two fragments, and 3.6 kb of L segment sequence in 7 fragments (Figure 2). The majority of arenavirus sequences were obtained from serum rather than tissue, potentially reflecting lower levels of competing cellular RNA in random amplification reactions.

Full genome characterization of a newly identified arenavirus

Sequence gaps between the aligned fragments were rapidly filled by specific PCR amplification with primers designed on the pyrosequence data at both, CU and CDC. Terminal sequences were added by PCR using a universal arenavirus primer, targeting the conserved viral termini (5'-CGC ACM GDG GAT CCT AGG C, modified from [30]) combined with 4 specific primers positioned near the ends of the 2 genome segments. Overlapping primer sets based on the draft genome were synthesized to facilitate sequence validation by conventional dideoxy sequencing. The accumulated data revealed a classical arenavirus genome structure with a bi-segmented genome encoding in an ambisense strategy two open reading frames (ORF) separated by an intergenic stem-loop region on each segment (Figure 2) (GenBank Accession numbers FJ952384 and FJ952385).

Our data represent genome sequences directly obtained from liver biopsy and serum (case 2), and from cell culture isolates obtained from blood at CDC (case 1 and 2), and from liver biopsies at NICD (case 2 and 3). No sequence differences were uncovered between virus detected in primary clinical material and virus isolated in cell culture at the two facilities. In addition, no changes were detected between each of the viruses derived from these first three cases. This lack of sequence variation is consistent with the epidemiologic data, indicating an initial natural exposure of the index case, followed by a chain of nosocomial transmission among subsequent cases.

Lujo virus (LUJV) is a novel arenavirus

Phylogenetic trees constructed from full L or S segment nucleotide sequence show LUJV branching off the root of the OW arenaviruses, and suggest it represents a highly novel genetic lineage, very distinct from previously characterized virus species and clearly separate from the LCMV lineage (Figure 3A and 3B). No evidence of genome segment reassortment is found, given the identical placement of LUJV relative to the other OW arenaviruses based on S and L segment nucleotide sequences. In addition, phylogenetic analysis of each of the individual ORFs reveals similar phylogenetic tree topologies. A phylogenetic tree constructed from deduced L-polymerase amino acid (aa) sequence also shows LUJV near the root of the OW arenaviruses, distinct from characterized species, and separate from the LCMV branch (Figure 3C). A distant relationship to OW arenaviruses may also be inferred from the analysis of Z protein sequence (Figure S1). The NP gene sequence of LUJV differs from other arenaviruses from 36% (IPYV) to 43% (TAMV) at the nucleotide level, and from 41% (MOBV/LASV) to 55% (TAMV) at the aa level (Table S1). This degree of divergence is considerably higher than both, proposed cut-off values within (<10–12%), or between (>21.5%) OW arenavirus species [31,32], and indicates a unique phylogenetic position for LUJV (Figure 3D). Historically, phylogenetic assignments of arenaviruses have been based on portions of the NP gene [1,33], because this is the region for which most sequences are known. However, as more genomic sequences have become available, analyses of full-length GPC sequence have revealed evidence of possible relationships between OW and NW

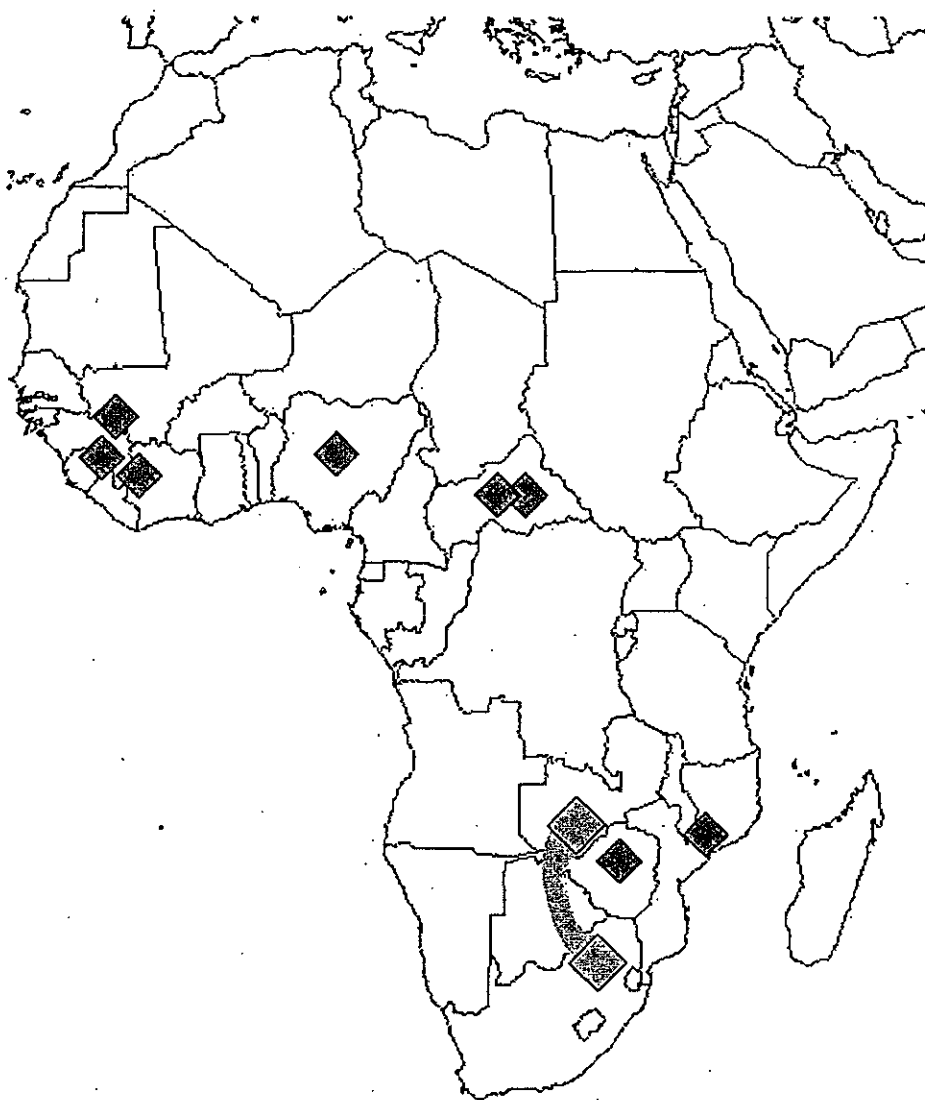


Figure 1. Geographic distribution of African arenaviruses. MOBV, MOPV, and IPPYV (blue) have not been implicated in human disease; LASV (red) can cause hemorrhagic fever. The origin of the LUJV index and secondary and tertiary cases linked in the 2008 outbreak are indicated in gold. doi:10.1371/journal.ppat.1000455.g001

arenaviruses not revealed by NP sequence alone [34]. Because G1 sequences are difficult to align some have pursued phylogenetic analyses by combining the GPC signal peptide and the G2 sequence for phylogenetic analysis [16]. We included in our analysis the chimeric signal/G2 sequence (Figure 3E) as well as the receptor binding G1 portion (Figure 3F); both analyses highlighted the novelty of LUJV, showing an almost similar distance from OW as from NW viruses.

Protein motifs potentially relevant to LUJV biology

Canonical polymerase domains pre-A, A, B, C, D, and E [35–37] are well conserved in the L ORF of LUJV (256 kDa, pI = 6.4; Figure 4). The Z-ORF (10.5 kDa, pI = 9.3) contains two late domain motifs like LASV; however, in place of the PTAP motif found in LASV, that mediates recognition of the tumor susceptibility gene 101, Tsg101 [38], involved in vacuolar protein sorting [39,40], LUJV has a unique Y₇₇REL motif that matches the YXXL motif of the retrovirus equine infectious anemia virus

[41], which interacts with the clathrin adaptor protein 2 (AP2) complex [42]. A Tsg101-interacting motif, P₉₀SAP, is found in LUJV in position of the second late domain of LASV, PPPY, which acts as a Nedd4-like ubiquitin ligase recognition motif [43]. The RING motif, containing conserved residue W₄₄ [44], and the conserved myristoylation site G₂ are present [45–47] (Figure 4). The NP of LUJV (63.1 kDa, pI = 9.0) contains described aa motifs that resemble mostly OW arenaviruses [48], including a cytotoxic T-lymphocyte (CTL) epitope reported in LCMV (GVYMGNL; [49]), corresponding to G₁₂₂VYRGNL in LUJV, and a potential antigenic site reported in the N-terminal portion of LASV NP (RKSKRND; [50]), corresponding to R₅₅KDKRND in LUJV (Figure 4).

The GPC precursor (52.3 kDa, pI = 9.0) is cotranslationally cleaved into the long, stable signal peptide and the mature glycoproteins G1 and G2 [51–54]. Based on analogy to LASV [55] and LCMV [56], signalase would be predicted to cleave between D₅₈ and S₅₉ in LUJV. However, aspartate and arginine

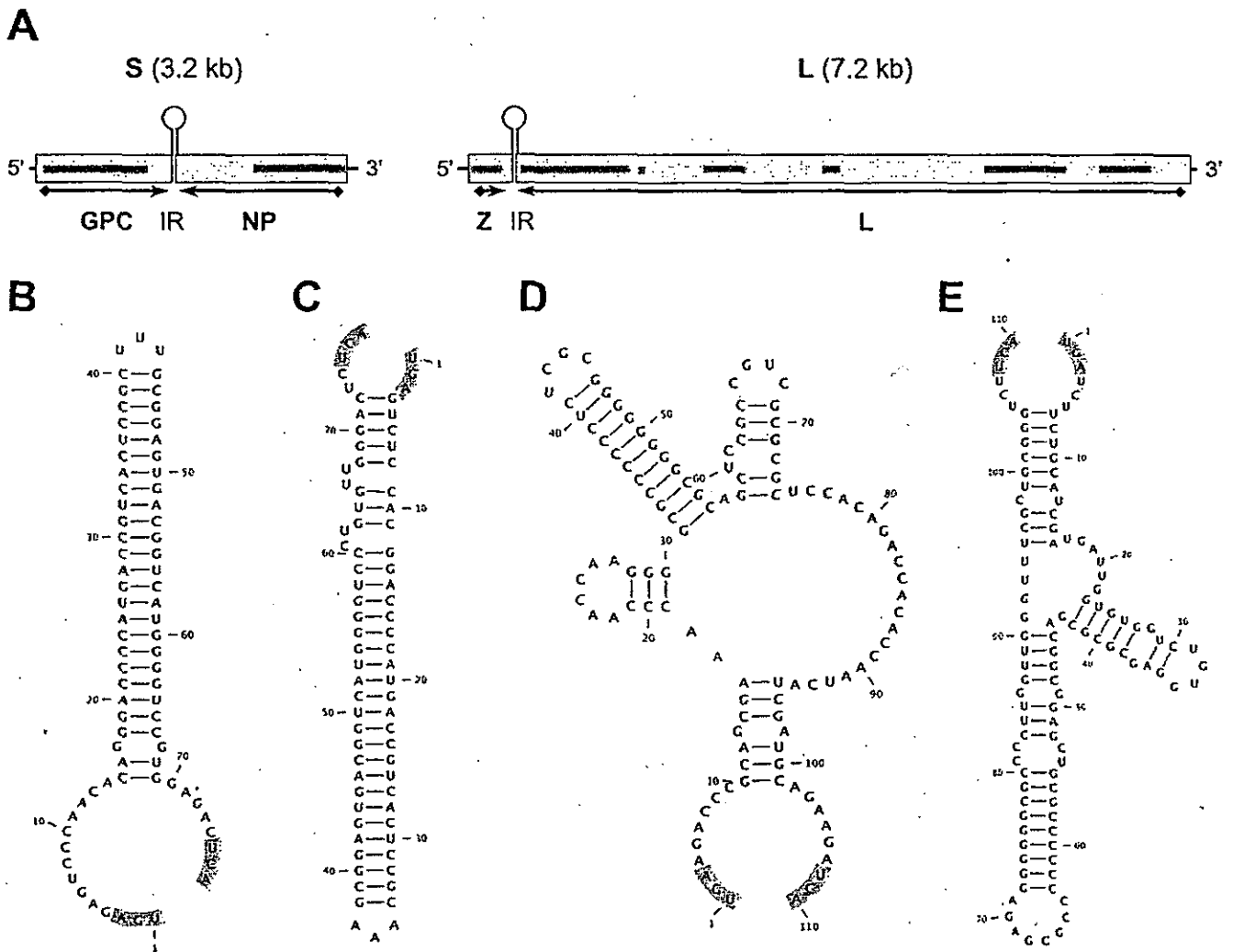


Figure 2. LUJV genome organization and potential secondary structure of intergenic regions. Open reading frames (ORF) for the glycoprotein precursor GPC, the nucleoprotein NP, the matrix protein analog Z, and the polymerase L, and their orientation are indicated (A); blue bars represent sequences obtained by pyrosequencing from clinical samples. Secondary structure predictions of intergenic regions (IR) for S (B, C) and L segment sequence (D, E) in genomic (B, D) and antigenomic orientation (C, E) were analyzed by mfold; shading indicates the respective termination codon (opal, position 1), and its reverse-complement, respectively. doi:10.1371/journal.ppat.1000455.g002

residues in the -1 and -3 positions, respectively, violate the $(-3, -1)$ -rule [57]; thus, cleavage may occur between S_{59} and S_{60} as predicted by the SignalP algorithm. The putative 59 aa signal peptide of LUJV displays a conserved G_2 , implicated in myristoylation in JUNV [58], however, it is followed in LUJV by a non-standard valine residue in position +4, resembling non-standard glycine residues found in Oliveros virus (OLVV [59]) and Latino virus (LATV; <http://www2.ncid.cdc.gov/arbocat/catalog-listing.asp?VirusID=263&SI=1>). Conservation is also observed for aa residues P_{12} (except Amapari virus; AMAV [60]), E_{17} [61] (except Pirital virus; PIRV [62]), and N_{20} in hydrophobic domain 1, as well as $I_{32}KGVFNLYK_{40}SG$, identified as a CTL epitope in LCMV WE ($I_{32}KAVYNFATCG$; [63]) (Figure 4).

Analogous to other arenaviruses, SKI-1/S1P cleavage C-terminal of $RKLM_{221}$ is predicted to separate mature G1 (162 aa, 18.9 kDa, $pI=6.4$) from G2 (233 aa, 26.8 kDa, $pI=9.5$) [52,53,64]. G2 appears overall well conserved, including the strictly conserved cysteine residues: 6 in the luminal domain, and 3 in the cytoplasmic tail that are included in a conserved zinc finger

motif reported in JUNV [65] (Figure 4). G2 contains 6 potential glycosylation sites, including 2 strictly conserved sites, 2 semi-conserved sites N_{335} (absent in LCMVs and Dandenong virus; DANV [19]) and N_{352} (absent in LATV), and 2 unique sites in the predicted cytoplasmic tail (Figure 4). G1 is poorly conserved among arenaviruses [16], and G1 of LUJV is no exception, being highly divergent from the G1 of the other arenaviruses, and shorter than that of other arenaviruses. LUJV G1 contains 6 potential glycosylation sites in positions comparable to other arenaviruses, including a conserved site $N_{93}HS$ (Figure 4), which is shifted by one aa in a motif that otherwise aligns well with OW arenaviruses and NW arenavirus clade A and C viruses. There is no discernable homology to other arenavirus G1 sequences that would point to usage of one of the two identified arenavirus receptors; Alpha-dystroglycan (α -DG) [66] that binds OW arenaviruses LASV and LCMV, and NW clade C viruses OLVV and LATV [67]; or transferrin receptor 1 (TFR1) that binds pathogenic NW arenaviruses JUNV, MACV, GTOV, and SABV [68] (Figure S2).

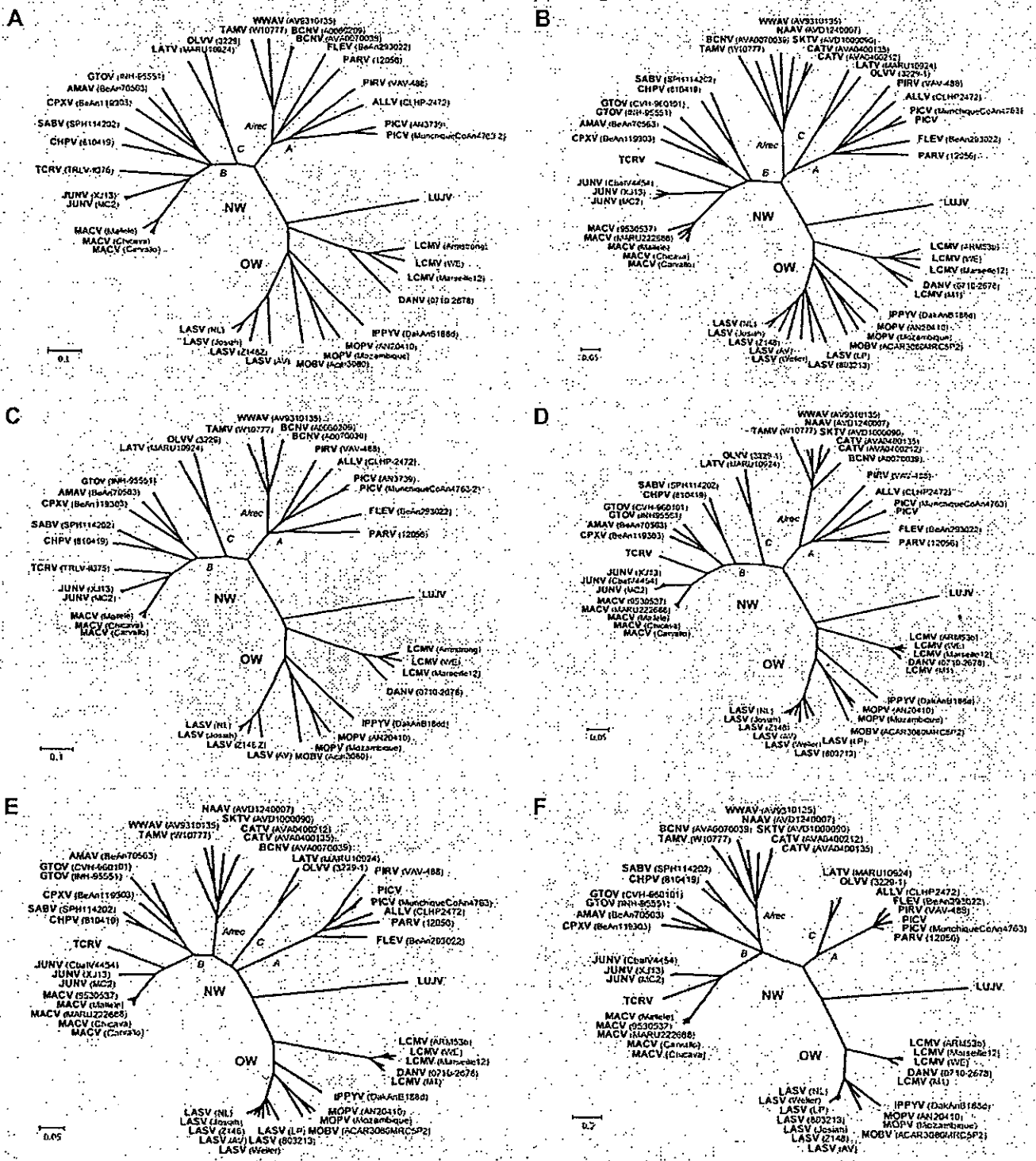


Figure 3. Phylogenetic analyses of LUJV. Phylogenetic relationships of LUJV were inferred based on full L (A) and S segment nucleotide sequence (B), as well as on deduced amino acid sequences of L (C), NP (D), Signal/G2 (E) and G1 (F) ORF's. Phylogenies were reconstructed by neighbor-joining analysis applying a Jukes-Cantor model; the scale bar indicates substitutions per site; robust bootstrap support for the positioning of LUJV was obtained in all cases (>98% of 1000 pseudoreplicates). GenBank Accession numbers for reference sequences are: ALLY CLHP2472 (AY216502, AY012687); AMAV BeAn70563 (AF512834); BCNV AVA0070039 (AY924390, AY922491), A0060209 (AY216503); CATV AVA0400135 (DQ865244), AVA0400212 (DQ865245); CHPV 810419 (EU, 260464, EU260463); CPXV BeAn119303 (AY216519, AF512832); DANV 0710-2678 (EU136039, EU136038); FLEV BeAn293022 (EU627611, AF512831); GTOV INH-95551 (AY358024, AF485258), CVH-960101 (AY497548); IPPYV DakAnB188d (DQ328878, DQ328877); JUNV MC2 (AY216507, D10072), XJ13 (AY358022, AY358023), CbaIV4454 (DQ272266); LASV LP (AF181853), 803213 (AF181854), Weller (AY628206), AV (AY179171, AF246121), Z148 (AY628204, AY628205), Josiah (U73034, J043204), NL (AY179172), 803213 (AF181854), Weller (AY628206), AV (AY179171, AF246121), Z148 (AY628204, AY628205), Josiah (U73034, J043204), NL (AY179172), 803213 (AF181853); MARU10924 (EU627612, AF485259); LCMV Armstrong (AY847351), ARM53b (M20869), Marseille12 (DQ228693, DQ286931), M1 (AB261991); MACV Carvallo (AY619642, AY619643), Chicava (AY624354, AY624355), Mallele (AY619644, AY619645), MARU22688

(AY922407), 9530537 (AY571959); MOBV ACAR3080MRC5P2 (DQ328876, AY342390); MOPV AN20410 (AY772169, AY772170), Mozambique (DQ328875, DQ328874); NAAV AVD1240007 (EU123329); OLVV 3229-1 (AY216514, U34248); PARV 12056 (EU627613, AF485261); PICV (K02734), MunchiqueCoAn4763 (EF529745, EF529744), AN3739 (AF427517); PIRV VAV-488 (AY216505, AF277659); SABV SPH114202 (AY358026, U41071); SKTV AVD1000090 (EU123328); TAMV W10777 (EU627614, AF512828); TCRV (J04340, M20304); WWAV AV9310135 (AY924395, AF228063). doi:10.1371/journal.ppat.1000455.g003

In summary, our analysis of the LUJV genome shows a novel virus that is only distantly related to known arenaviruses. Sequence divergence is evident across the whole genome, but is most pronounced in the G1 protein encoded by the S segment, a region implicated in receptor interactions. Reassortment of S and L segments leading to changes in pathogenicity has been described in cultured cells infected with different LCMV strains [69], and between pathogenic LASV and nonpathogenic MOPV [70]. We find no evidence to support reassortment of the LUJV L or S genome segment (Figure 3A and 3B). Recombination of glycoprotein sequence has been recognized in NW arenaviruses [14,16,33,34,71–73], resulting in the division of the complex into four sublineages: lineages A, B, C, and an A/recombinant lineage that forms a branch of lineage A when NP and L sequence is considered (see Figure 3C and 3D), but forms an independent branch in between lineages B and C when glycoprotein sequence is considered (see Figure 3D). While recombination cannot be excluded in case of LUJV, our review of existing databases reveals no candidate donor for the divergent GPC sequence. To our knowledge is LUJV the first hemorrhagic fever-associated arenavirus from Africa identified in the past 3 decades. It is also the first such virus originating south of the equator (Figure 1). The International Committee on the Taxonomy of Viruses (ICTV) defines species within the *Arenavirus* genus based on association with a specific host, geographic distribution, potential to cause

human disease, antigenic cross reactivity, and protein sequence similarity to other species. By these criteria, given the novelty of its presence in southern Africa, capacity to cause hemorrhagic fever, and its genetic distinction, LUJV appears to be a new species.

Materials and Methods

Sequencing

Clinical specimens were inactivated in TRIzol (liver tissue, 100 mg) or TRIzol LS (serum, 250 µl) reagent (Invitrogen, Carlsbad, CA, USA) prior to removal from BSL-4 containment. Total RNA extracts were treated with DNase I (DNA-free, Ambion, Austin, TX, USA) and cDNA generated by using the Superscript II system (Invitrogen) and 100–500 ng RNA for reverse transcription primed with random octamers that were linked to an arbitrary, defined 17-mer primer sequence [74]. The resulting cDNA was treated with RNase H and then randomly amplified by the polymerase chain reaction (PCR; [75]); applying a 9:1 mixture of a primer corresponding to the defined 17-mer sequence, and the random octamer-linked 17-mer primer, respectively [74]. Products >70 base pairs (bp) were selected by column purification (MinElute, Qiagen, Hilden, Germany) and ligated to specific linkers for sequencing on the 454 Genome Sequencer FLX (454 Life Sciences, Branford, CT, USA) without fragmentation of the cDNA [19,76,77]. Removal of primer sequences, redundancy filtering,

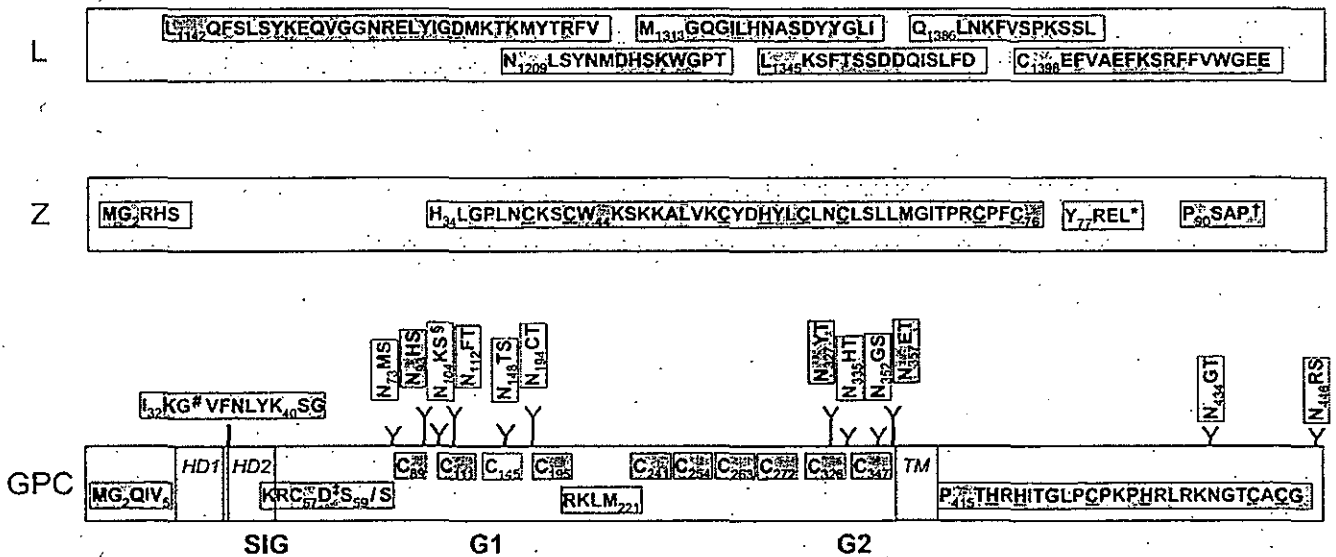


Figure 4. Schematic of conserved protein motifs. Conservation of LUJV amino acid motifs with respect to all other (green highlight), to OW (yellow highlight), or to NW (blue highlight) arenaviruses is indicated; grey highlight indicates features unique to LUJV. Polymerase motifs pre-A (L1142), A (N1209), B (M1313), C (L1345), D (Q1386), and E (C1398) are indicated for the L ORF; potential myristoylation site G₂, the RING motif H₂₄/C₇₆, and potential late domains YXXL and PSAP are indicated for the Z ORF; and myristoylation site G₂, posttranslational processing sites for signalase (S₅₉/S₆₀) and S1P cleavage (RKL M₂₂₁), CTL epitope (I₃₂), zinc finger motif P₄₁₅/G₄₄₀, as well as conserved cysteine residues and glycosylations sites (Y) are indicated for GPC. * late domain absent in NW viruses and DANV; † PSAP or PTAP in NW viruses, except in PIRV and TCRV (OW viruses: PPPY); # G in all viruses except LCMV (=A); ‡ D in NW-clade A only; § conserved with respect to OW, and NW clade A and C; HD, hydrophobic domain; TM, transmembrane anchor. doi:10.1371/journal.ppat.1000455.g004