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Viral Hemorrhagic Fever Diagnostics

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There are 4 families of viruses that cause viral hemorrhagic fever (VHF), including *Filoviridae*. Ebola virus is one virus within the family *Filoviridae* and the cause of the current outbreak of VHF in West Africa. VHF-endemic areas are found throughout the world, yet traditional diagnosis of VHF has been performed in large reference laboratories centered in Europe and the United States. The large amount of capital needed, as well as highly trained and skilled personnel, has limited the availability of diagnostics in endemic areas except in conjunction with governmental and nongovernmental entities. However, rapid diagnosis of VHF is essential to efforts that will limit outbreaks. In addition, increased global travel suggests VHF diagnoses may be made outside of the endemic areas. Thus, understanding how to diagnose VHF is imperative for laboratories worldwide. This article reviews traditional and current diagnostic modalities for VHF.

Keywords. viral hemorrhagic fever; diagnostics; Ebola.

INTRODUCTION

Viral hemorrhagic fevers (VHFs) are caused by 4 families of viruses (*Arenaviridae*, *Bunyaviridae*, *Filoviridae*, and *Flaviviridae*), with multiple genera and species causing disease (Table 1). All 4 viral families are single-stranded RNA viruses that have a lipid envelope, which makes them susceptible to detergents and environments with low pH; however, they are stable in blood and cold storage [1]. The 4 families of viruses are zoonoses, with reservoirs recognized for all species except for Ebola virus (EBOV). Fruit bats are assumed to be the reservoir, but only serological evidence and viral sequences of EBOV have been detected [2]. Arenaviruses, Crimean-Congo hemorrhagic fever virus (CCHFV), and filoviruses can be transmitted from human to human by contact with blood and other body fluids, potentially expanding exposed individual cases into epidemic outbreaks, including the current EBOV disease outbreak in western Africa [3].

Signs and Symptoms of Viral Hemorrhagic Fevers

VHFs were initially classified together due to the common signs and symptoms of the disease processes. Patients may initially present with fever and general malaise similar to what is seen with other common tropical diseases, including malaria and typhoid. The rarity of VHF, along with indiscriminating symptoms, make diagnosing VHF problematic, even after the fulminant disease process has begun. Distinguishing VHF from other tropical diseases is important, not only for isolation and infection control procedures, which can help stop the spread of VHF, but for proper management of VHF and/or coexisting diseases.

Several groups have looked at common laboratory parameters to determine if specific laboratory values may help delineate VHF from other diseases. Blood urea nitrogen and creatinine were elevated in patients with hemorrhagic fever with renal syndrome caused by Hantaan virus and Seoul virus, while Huaiyangshan hemorrhagic fever virus–infected patients were found to have leukopenia and increased creatine kinase and alanine aminotransferase (ALT) [4]. Leukopenia, thrombocytopenia, and increased ALT and aspartate aminotransferase (AST) have been observed in patients with CCHFV [5], Sudan virus disease, and EBOV disease [6, 7]. Hematology parameters in areas endemic for Dengue hemorrhagic fever indicated that platelet counts of $<100 \times 10^9$/L, a prolonged activated partial thromboplastin time, normal prothrombin time, and elevated aspartate aminotransferase and ALT are useful in evaluating the likelihood of Dengue hemorrhagic fever [9]. Other studies have shown a high C-reactive protein concentration (>5 mg/L) is more predictive of malaria than Dengue hemorrhagic fever [10]. These laboratory values are not highly specific for diagnosing VHF. However, in areas where direct diagnostic techniques for VHF are not readily available, laboratory markers may offer guidance and direction for identifying patients with VHF. Higher sensitivity and specificity in detecting VHF is demonstrated in laboratory methods that directly detect virus or humoral immune response to virus from patient specimens. Serological markers for measuring virus-specific immunoglobulin M (IgM) and IgG in patient serum offer a potential delineator of disease that lacks the technical and logistical restraints of the gold standard of molecular detection. This has been effectively demonstrated for Lassa and the early detection of IgM for

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Table 1. Viral Hemorrhagic Fevers in Humans

Family	Genus	Species	Virus Member Abbreviation
Arenaviridae			
	Mammarenavirus (Old World)	*Lassa mammarenavirus*	LASV
		Lujo mammarenavirus	LUJV
	Mammarenavirus (New World)	*Chapare mammarenavirus*	CHPV
		Guanarito mammarenavirus	GTOV
		Junín mammarenavirus	JUNV
		Machupo mammarenavirus	MACV
		Sabiá mammarenavirus	SABV
Bunyaviridae			
	Nairovirus	*Crimean-Congo hemorrhagic fever virus*	CCHFV
	Phlebovirus	*Henan fever virus*[a]	HNFV
		Huaiyangshan virus[a]	HYSV
		Rift Valley fever virus	RVFV
		Severe fever and thrombocytopenia syndrome virus[a]	SFTSV
	Hantavirus	*Dobrava-Belgrade virus*	DOBV
		Hantaan virus	HTNV
		Puumalavirus	PUUV
		Saaremaa virus	SAAV
		Seoul virus	SEOV
		Sin Nombre virus	SNV
		Tula virus	TULV
Filoviridae			
	Ebolavirus	*Bundibugyo ebolavirus*	BDBV
		Sudan ebolavirus	SUDV
		Taï Forest ebolavirus (formerly Cote d'Ivoire)	TAFV
		Zaire ebolavirus	EBOV
	Marburgvirus	*Marburg marburgvirus*	MARV
Flaviviridae			
	Flavivirus	*Dengue virus*	DENV-1-4
		Kyasanur forest disease virus	KFDV
		Omsk hemorrhagic fever virus	OHFV
		Yellow fever virus	YFV

[a] Virus not classified as a species in the International Committee of Taxonomy of Viruses, 2014 release [8].

Lassa virus (LASV) and IgG for Rift Valley fever virus (RVFV) infections [11, 12].

DIAGNOSTIC METHODS

Laboratory Diagnosis

Laboratory diagnosis of VHF has traditionally taken place in highly specialized reference laboratories. These laboratories have been classified with biosafety levels (BSL) ranging from 1 to 4 by the World Health Organization. The laboratories are categorized according to the laboratory design, containment facility, and handling of biological agents classified into 4 specific risk groups (RG 1–4) [13]. RG-1 contains pathogens not likely to cause disease in healthy individuals. Organisms that can cause disease in humans but are not likely to be a serious threat to laboratory workers are in RG-2. Known treatment regimens or preventive measures are available for these organisms. RG-3 contains organisms that can cause serious diseases in humans but do not ordinarily spread from one person to another. These pathogens have known prevention and treatment measures. Lastly, pathogens that are likely to cause serious disease in humans and can be readily transmitted from one person to another are classified as RG-4 agents. Usually no approved treatment or preventive measures are available for this RG [13]. According to the Centers for Disease Control and Prevention, the VHF viruses are members of RG-2 through RG-4 [14]. Dengue virus (DENV) is the only organism that does not fall into BSL-3 or BSL-4 laboratory practices. There are a limited number of BSL-4 laboratories throughout Europe, the United States, and Africa, and the distance of BSL-4 laboratories from endemic areas often lengthens time to diagnosis when most needed. Some countries, but not all with endemic VHF, often make diagnoses in their laboratories, but they are not BSL-4 certified. Financial means and lack of highly trained personnel, equipment, and supply lines that would ensure maintenance of a cold-chain system limit the capabilities of these laboratories. Personal protective equipment can be modified to be effective in these resource-limited environments.

Virus Culture and Electron Microscopy

Culture of live agents was considered the gold standard but is rapidly being challenged by polymerase chain reaction (PCR) and next generation sequencing [15]. Culture of live agents remains helpful for characterization of new or divergent viruses, but next generation sequencing can also aid in identifying these viruses. Electron microscopy (EM) may be used to identify virus based on structural characteristics from clinical materials or culture [16]. Agent-specific antibodies in conjunction with EM may be used to further identify or classify an agent and to increase the sensitivity of this method, depending on the protocol used. Culture of live virus and preparation of samples for EM requires access to highly specialized laboratories, and completion of these procedures can be lengthy, taking weeks for the virus to grow and additional time to perform the microscopy. As a result, these methods are no longer used as a first line of diagnosis in many instances.

Nucleic Acid Detection

Nucleic acid detection has become a common diagnostic tool for identifying viral diseases, including VHF. Such detection surpasses the limit of detection for many culture methods and eliminates the need for the virus to be viable at the time of testing. Nucleic acid extraction techniques vary, but 2 common reagents—TRIzol (Life Technologies, Grand Island, New York) reagent and AVL plus ethanol buffer (Qiagen, Germany)—were shown to inactivate filoviruses, flaviviruses, and bunyaviruses [17], decreasing exposure to live virus for laboratory personnel once placed into these buffers. In addition, another group found their system (Tripure, Roche, Switzerland) could also inactivate filoviruses when used per manufacturer guidelines [18]. The RNA extracted after these methods are performed is fairly stable, which facilitates the ease of shipping and transport to other laboratories.

Reverse transcription PCR (RT-PCR) has become the cornerstone for molecular diagnosis, and RT-PCR assays have been developed for the majority of VHF-associated viruses [19–22]. Detecting 1 pathogen at a time using these assays has had limitations in areas where more than 1 VHF pathogen is endemic. Multiplex assays for VHF were first described in 2002 with the ability to detect EBOV, Marburg virus (MARV), LASV, CCHFV, RVFV, DENV, and yellow fever virus (YFV) [22]. Multiplex assays have now been developed for a range of VHFs with sensitivity and specificity comparable to other RT-PCR assays; 1 study showed a limit of detection ranging from 1×10^{-2} to 1×10^{5} copies/mL [23] and another study detected viruses at 0.0001 to 10 plaque forming units (PFU)/mL [24]. Assays available at standard reference laboratories, such as Associated Regional and University Pathologists Laboratories (Salt Lake City, Utah) and Mayo Medical Laboratories (Rochester, Minnesota), include serologic testing for DENV and Hanta virus and RT-PCR for DENV.

Microarrays are a specialized type of multiplex PCR that uses a solid matrix spotted with oligonucleotides that represent specific genes of different organisms. An unknown fluorescently labeled DNA anneals and releases a positive signal that is compared with the intensity of known positive signals. Microarrays have a high specificity and sensitivity to detect all 4 virus families that encompass VHF [25]. FilmArray (Biofire, Salt Lake City, Utah) has developed a qualitative biodefense panel for detecting EBOV and MARV [26] that has the capability of rapid field diagnostics. Implementation of this panel in the field would necessitate investment in capital equipment related to this test, as well as an investment in the test pouches, which are used once and discarded. The advantages of the test pouches are that they are lyophilized and stable; the disadvantage is that the pouches have a short shelf life (6 months after the date of manufacture).

Field Diagnostics

In outbreak settings, results from RT-PCR were traditionally sent to international reference laboratories [27] with a long turnaround time. Towner et al developed a protocol for field diagnostics in a mobile laboratory unit using quantitative RT-PCR [18]. The mobile laboratory unit (which was co-located with the hospital) was implemented during an outbreak of MARV in Angola and had the ability to diagnose patients in less than 4 hours [27]. Serum or plasma, collected as whole blood in EDTA-coated tubes (heparin is avoided since it inhibits PCR), has been the specimen of choice for nucleic acid testing; however, in some countries, phlebotomy is not culturally acceptable [27, 28]. Oral and nasal swabs have been used but can be insensitive depending on the variability in the swabbing technique that resulted from lack of training. Suspected patients who are negative by swab still need to be tested by more sensitive quantitative molecular testing [27, 28]. Use of rapid diagnostic tests in outbreak settings makes contact tracing in a timely manner. Rather than waiting days for results after shipping specimens to a laboratory and waiting for transmission of results back to the field, results are available within a few hours. This allows for more rapid identification of individuals who have had contact with patients with disease and helps prevent further transmission [27]. In addition to the MARV outbreak, a portable thermocycler helped diagnose patients in as little as 3 hours of arrival to a hospital during a RVFV outbreak, leading to quick and appropriate treatment for patients with disease [29].

Another molecular assay with potential for field diagnostics is loop-mediated isothermal amplification (LAMP). This method amplifies nucleic acids using 2 nested primers, and the reaction takes place at a single temperature (65°C), omitting the need for expensive thermocyclers [30, 31]. The product can be measured in real time by turbidometry or through an intercalating dye [30]. One LAMP assay that has been developed for RVFV produces results within 2 hours, including RNA

extraction [31]. LAMP assays have been developed for detection of severe fever and thrombocytopenia syndrome virus, YFV, CCHFV, MARV, DENV, and EBOV [32–37]. LAMP and other RT-PCR assays are becoming more widely available; however, the capability for diagnostic testing with RT-PCR is not always an option in endemic areas, due to lack of funding and infrastructure.

Diagnostics that use antigen detection by immunochromatography require little training, use minimal amounts of patient sample, and do not require electricity. These antigen assays are also less susceptible to viral drift or evolution, since accumulated mismatches in the RT-PCR primers and probes can impact the performance of molecular tests. An immunofiltration test is available for EBOV and can detect viral proteins from urine within 30 minutes. The sensitivity is less than that of RT-PCR but comparable to that of other available antigen tests [38]. A rapid diagnostic kit for DENV that can detect both the NS1 antigen and antibodies to DENV by immunochromatography was tested in Cambodia during an outbreak and had high sensitivity but low negative predictive value [39]. Immunochromatographic techniques have also been developed for RVFV and Hanta virus but have not been used in diagnosing human infections [40, 41]. A lateral flow immunoassay has been developed and used in diagnosing LASV when patients are viremic [42]. Currently, an emergency use authorization has been issued for the ReEBO Antigen Rapid (15 minutes) Test (Corginex, Broomfield, Colorado), a lateral flow assay developed for detection of EBOV and Sudan ebolavirus VP40 antigen with a sensitivity found acceptable in preliminary studies (Table 2) [43–45]. The Defense Science and Technology Laboratory of the United Kingdom deployed a lateral flow assay in the current EBOV disease outbreak in Sierra Leone [46].

Other Diagnostic Modalities

Enzyme-linked immunosorbent assay is the most common method for detecting IgM and IgG antibodies. Serology is not useful in diagnosing acute VHF illness, as the presence of IgM antibodies can represent different stages of disease or a prior symptomatic or asymptomatic infection. The diagnosis of recent VHF may need additional blood draws to determine if the patient has an increasing IgM titer [47]. In endemic areas, a positive IgG may mean little in trying to diagnose an acute infection [48]. Serologic diagnosis often lacks specificity with high cross-reactivity between closely related viruses, especially within *Bunyaviridae* and *Flaviviridae* [49]. Despite the issues with serologic methods, including known cross-reactivity among the family of viruses [50], serology is the gold standard and the most common method for diagnosing bunyaviruses, due to short viremic periods and rapid clearance of the virus by the immune system [47, 49].

Immunohistochemistry

Immunohistochemical staining of skin biopsies infected with EBOV described by Zaki et al was concordant with other diagnostic modalities used for confirming EBOV disease [51].

Table 2. Ebolavirus Assays With Emergency Use Authorization

Test	Manufacturer	Target	Method of Detection	Machine	EUA (Date)	EUA Link
EZ-1	DOD	GP	qRT-PCR	ABI 7500, light cycler, JBAIDS	Yes (10 October 2014)	EZ-1.pdf
CDC VP40	CDC	VP40	qRT-PCR	ABI 7500 fast DX	Yes (10 October 2014)	CDC VP40.pdf
CDC NP	CDC	NP	qRT-PCR	ABI 7500 fast DX	Yes (10 October 2014)	CDC NP.pdf
Biothreat E-test	Biofire	Not identified	qRT-PCR	Film array instrument	Yes (25 October 2014)	Biothreat E-test.pdf
NGDS BT-E Assay	Biofire	Not identified	qRT-PCR	Film array instrument	Yes (25 October 2014)	NGDS BT-E Assay.pdf
RealStar Ebolavirus Kit 1.0	Altona Diagnostics GMBH	Ebola L gene (RDRP)	qRT-PCR	ABI 7500, LightCycler 480, CFX96	Yes (26 November 2014)	RealStar Ebolavirus Kit 1.0.pdf
LightMix Ebola Zaire rRT-PCR	Roche	Ebola L gene (RDRP)	qRT-PCR	LightCycler 480 instrument and/or COBAS z 480 analyzer	Yes (23 December 2014)	LightMix Ebola Zaire rRT-PCR.pdf
ReEBOV Antigen Rapid Test	Corgenix Inc.	VP40	Lateral flow immunoassay	N/A	Yes (24 February 2015)	ReEBOV Antigen Rapid Test.pdf

US Food and Drug Administration EUA approvals: http://www.fda.gov/MedicalDevices/Safety/EmergencySituations/ucm161496.htm.

Abbreviations: CDC, Centers for Disease Control and Prevention; DOD, Department of Defense; EUA, emergency use authorization; GP, glycoprotein; JBAIDS, Joint Biological Agent Identification and Diagnostic System; NP, nucleoprotein; qRT-PCR, quantitive real-time reverse transcription polymerase chain reaction; RDRP, RNA-dependent RNA polymerase; VP40, viral matrix protein.

A skin biopsy is fixed in formalin, which renders the virus noninfectious. The specialized laboratory processes required for this procedure do not allow for a rapid diagnosis but could be used as a surveillance tool or an alternative to autopsy in deceased patients.

LABORATORY INFECTION PREVENTION

Diagnosing VHF can cause anxiety for laboratory personnel [52], who fear they will become infected while working with contagious laboratory specimens. In areas with appropriate resources, including heat and detergent, laboratory specimens can be inactivated by gamma irradiation [53] and with RNA extraction techniques [17]. Even without inactivation, procedures for proper use of protective equipment and training have helped to stop transmission during VHF outbreaks [54] and, hence, to limit transmission to laboratory personnel.

CONCLUSION

Molecular detection by RT-PCR in blood is the most sensitive assay for diagnosing known VHF in symptomatic patients. However, these assays have traditionally not been widely available outside of BSL-4 reference laboratories. Recent advances in field testing have made assays for VHF available to those in endemic areas but still require capital investment, highly trained personnel, and advanced technology from outside nations. Future advancements in diagnostic testing may occur as a result of biomarkers or other host signatures that can predict active disease [55].

Future test development needs to include increasing the sensitivity and specificity of rapid testing that can be performed in resource-limited settings, where humidity and temperature stability are not favorable. These tests are critical to rapid triage and care of patients, where individuals with a positive result can be quarantined and other individuals with compelling symptoms can be retested or undergo more sensitive molecular testing. These approaches will ultimately lead to faster contact tracing and containment of disease outbreaks.

Notes

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