Laboratory diagnosis of Ebola and Marburg hemorrhagic fever.

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Introduction

Identification of viral hemorrhagic fever (VHF) caused by Ebola virus (EBOV) and Marburg virus (MARV), family Filoviridae (5) may be difficult due to a wide variety of infectious diseases causing similar clinical symptomatology. The most common causes of severe, acute, febrile diseases in Filovirus endemic areas are malaria and typhoid fever. A wide range of infectious diseases must be considered next, such as shigellosis, meningococcal sepsicaemia, plague, leptospirosis, anthrax, relapsing fever, typhus, murine typhus, yellow fever, Chikungunya fever, Dengue fever and fulminant viral hepatitis. Travel, treatment in local hospitals, contact to sick persons or wild and domestic animals, particularly monkeys, are useful historical features, especially in returning travelers from Africa. Diagnosis of single cases is extremely difficult, but the occurrence of clusters of cases with prodromal fever followed by cases of hemorrhagic diatheses and person-to-person transmission are suggestive of VHF, and containment procedures have to be initiated. If filoviral VHF prostration, lethargy, wasting, and diarrhoea seem to be more severe than observed in patients with other VHFs. If present, the macular rash is characteristic and useful in narrowing the differential diagnosis.

Current diagnostics

Despite all the achievements in laboratory diagnostics in the past decades, it should be kept in mind that the diagnosis of EBOV and MARV infections will initially have to be based on clinical assessment. For this purpose contingency plans should be developed that are still missing in many, particularly developing countries. As clinical microbiology and public health laboratories are not generally equipped for diagnosis of VHF, particularly Filovirus VHF, it is necessary that samples are sent to national and/or international reference laboratories capable of performing the required assays. In addition, many nations encounter difficulties in sample transport, which can cause substantial delays in laboratory response. Once samples are received, laboratory response is fairly efficient with results expected within 24 to 48 hours.
Early laboratory diagnosis of EBOV and MARV infections is most desirable because of current implications in case patient management and public health interventions. There is currently no therapeutic treatment for Filovirus infections but this may become available in future (2, 7). If treatment is to be implemented, rapid, sensitive, and quantitative laboratory assays will be needed especially to initiate and evaluate treatment options such as anticoagulant therapy (7).

Laboratory diagnosis of Filovirus infections can basically be achieved in two ways: Measurement of the host-specific immune responses to the infection and detection of viral particles or particle components in infected individuals (table I). Today, reverse transcriptase-polymerase chain reaction (RT-PCR) (3, 28, 29, 31) and antigen detection “Enzyme-linked Immunosorbent Assay” (ELISA) (12, 17, 18, 21, 25) are the primary test systems to diagnose an acute infection. For antibody detection the most commonly used assays are direct IgG and IgM ELISAs and IgM capture ELISA (1, 10, 11, 17, 19, 22).

Confirmatory tests include western blot (1, 4) and the indirect immunofluorescence assay (IFA) on acetone-fixed infected cells inactivated by γ-radiation (13, 15, 16, 32). Due to relatively high viremia levels in humans, electron microscopy has been particularly useful in diagnosis of Filovirus infections (8, 9, 14, 23, 30). Viral structures can be directly visualized in serum, in culture fluid from initial passage cell cultures by negative staining, and in thin sections of any infected material. Immunohistochemistry (IHC) on formalin-fixed material and paraffin-embedded tissues can be used for detection of filoviruses (14) as well as immunofluorescence (IF) on impression smears of tissues (26). For non-outbreak surveillance, IHC on formalin-fixed biopsy/autopsy material is available for filoviruses and has several advantages including its simplicity, specificity and the lack of any need for enhanced biocontainment (33).

Virus isolation from serum or other clinical material should always be attempted. The most commonly used cell lines for isolation are Vero cells (clone E6) and MA-104 cells (both monkey kidney cells). However, most filoviruses do not cause extensive cytopathic effect on primary isolation. Guinea pigs can be used for primary isolation of those filoviruses that initially do not grow well in tissue culture. Several passages are usually required to produce a uniformly fatal disease.

### Table I.

<table>
<thead>
<tr>
<th>Test</th>
<th>Target</th>
<th>Source</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymerase chain reaction (PCR)</td>
<td>viral nucleic acid</td>
<td>blood, serum, tissues</td>
<td>rapid and sensitive, but requires special equipment</td>
</tr>
<tr>
<td>Antigen ELISA</td>
<td>viral antigen</td>
<td>blood, serum, tissues</td>
<td>rapid and sensitive, but requires special equipment</td>
</tr>
<tr>
<td>Immunohistochemistry</td>
<td>viral antigen</td>
<td>tissues (e.g., skin, liver)</td>
<td>inactivated material, but requires time</td>
</tr>
<tr>
<td>Fluorescence assay (FA)</td>
<td>viral antigen</td>
<td>tissues (e.g., liver)</td>
<td>rapid and easy, but interpretation is subjective</td>
</tr>
<tr>
<td>Electron microscopy</td>
<td>viral particle</td>
<td>blood, tissues</td>
<td>unique morphology (immunoscreening possible), but insensitive and requires expensive equipment</td>
</tr>
<tr>
<td>Indirect immunofluorescence assay</td>
<td>virus-specific antibodies</td>
<td>serum</td>
<td>simple to perform, but prone to non-specific positives and subjective interpretation</td>
</tr>
<tr>
<td>Enzyme linked immunosorbent assay</td>
<td>virus-specific antibodies</td>
<td>serum</td>
<td>specific and sensitive, but initial response slower than IFA</td>
</tr>
<tr>
<td>Immunoblot (Westernblot)</td>
<td>virus-specific antibodies</td>
<td>serum</td>
<td>protein-specific, but interpretation sometimes difficult</td>
</tr>
<tr>
<td>Virus isolation</td>
<td>viral particle</td>
<td>blood, tissues</td>
<td>virus available for studies, but needs biocontainment and requires time</td>
</tr>
</tbody>
</table>

### Interpretation of diagnostic test results

Of the available techniques for diagnosis, antigen-capture ELISA and RT-PCR are today the most useful for making a diagnosis in an acute clinical setting. Viral antigen/nucleic acid can be detected in blood from day 3 up to 7 to 16 days post onset of symptoms (27). RT-PCR assays seem to be favored by many investigators since BSL-4 biocontainment is not necessary after proper inactivation as well as the sensitivity/specifcity and rapidity of the technique. However, the diagnosis of index cases of outbreaks or of single imported cases should not be solely based on RT-PCR to avoid the problem of cross-contamination and, thus, false-positives. Confirmation by an independent assay such as antigen capture ELISA should always be attempted. When case confirmatory techniques and biocontainment (virus isolation) are not available, RT-PCR on an independent target gene and/or independent sample should be the minimum confirmation. In such instances it may be useful to seek confirmation through another reference laboratory.

Serology can be useful for confirmation but it should be kept in mind that negative serology is not exclusive since filovirus-infected individuals often die without a proper humoral immune response. Based on past investigations, IgM antibodies can appear as early as two days post onset of symptoms and disappear between 30 and 168 days after infection. IgG-specific antibodies develop between day 6 and 18 after onset and persist for many years (27). Rising IgM or IgG titer constitutes a strong presumptive diagnosis. However, a single positive result should be confirmed on a follow-up sample preferentially taken a week apart. Decreasing IgM and/or increasing IgG titer (four-fold) in successive paired sera are highly suggestive of a recent infection.

Standardization and evaluation of diagnostic procedures for EBOV and MARV is difficult because of the restricted
availability of virologic and clinical material. Recently, the “European Network for Imported Viral Diseases” (ENIVD) provided an external quality assurance for Filovirus PCR diagnostic procedures (24). Though most laboratories in this study showed reasonable abilities to detect filoviruses by PCR technology, a small but significant fraction of laboratories demonstrated rather poor sensitivities. A quality assurance panel for EBOV diagnostics was also performed by the “International High Security Laboratory Network” (IHSLN) (IHSLN, unpublished data) and revealed similar results. Thus, continued and extended quality assurance studies are required to maximize the robustness of Filovirus diagnostic procedures.

Field diagnostics

The recent years have been marked by repeated outbreaks of EBOV in several countries of Central Africa (6). Often these outbreaks occur in remote sites where sophisticated medical support systems are limited and timely diagnostic services are extremely difficult to provide. Provision of a fieldable laboratory to provide basic diagnostics for filoviruses, and other agents that may be confounding to the diagnosis, could help address the management of patients specifically and the outbreak in general. The development of truly portable real-time thermocyclers and simple, fieldable immunological assays has made the provision of a field diagnostic laboratory a reasonable undertaking.

As it might not be possible to relocate an entire laboratory to remote settings, it is necessary to bring the equipment essential to provide a safe work environment while completing the necessary testing. The initial and most important task is to minimize exposure for workers. If time and logistics permit, this can be ideally accomplished using a portable class III biosafety cabinet. This allows for safe handling of samples until the infectious agents are inactivated or packaged appropriately for shipping. Although portable, these units are often too large to travel as checked baggage on commercial flights and would need to be shipped cargo or by charter, often too large to travel as checked baggage on commercial flights and would need to be shipped cargo or by charter, which can cause delays. Alternatively, personal protective equipment (PPE) similar to that used by the isolation ward which can cause delays. Alternatively, personal protective equipment (PPE) similar to that used by the isolation ward can be used to protect workers while handling infectious material. Clinical and other specimens can be safely inactivated for serological analysis by heat in the presence of appropriate ionic or non-ionic detergents. We have used a final concentration of 0.2% sodium dodecyl sulphate (SDS)/0.1% Tween 20 and heat treatment of 60°C for 15 minutes to treat samples prior to ELISA based assays and 1% SDS prior to immunofiltration based antigen detection assays.

For molecular diagnostics, samples are commonly inactivated using a guanidine thiocyanate buffers supplied with commercial RNA isolation kits. Real-time PCR based assays run on machines designed to be portable are well suited to these environments and can provide extremely sensitive tests in a very short time. In addition to confirming the presence of a virus in patient samples, real-time tests also provide a quantitative assay that may be of use when viral load determination is necessary as in trials of antivirals or other treatment regimes. Tests for other agents that may be confounding and mistaken originally as the causative agent (e.g., Plasmodium sp.) should also be included in the reagent inventory. The introduction of real-time RT-PCR allows assays to be carried out with minimal manipulation and equipment and can provide results in less than two hours.

The first step of molecular diagnostic techniques requires the extraction/purification of the RNA from the sample, which can then serve as a template for conversion from RNA to DNA by the action of a reverse transcriptase. This is followed by the specific amplification of viral sequences in the PCR assay. Several methods and chemistries are available for the amplification reaction, which also allow monitoring the accumulation of product for real-time. Our approach is using two primers directed against well-conserved regions of the Filovirus genome and monitoring for the accumulation of amplification products using SYBR Green I, a dye which upon binding non-specifically to double-stranded DNA, results in an increase in fluorescence at 530 nm. As this dye will non-specifically bind to all double-stranded DNA produced during the amplification process, the presence of specific Filovirus product must be determined by melt point analysis of the products carried out at the end of the amplification (figure 1A, 1B). The primers (EBsp5 – 5’ TTYCCTAGCAAYATGATGG, EBsp3 – 5’TATAATTACACTGACATGCAT) detect an approximately 250 bp region of the glycoprotein gene of EBOV species. The electrical power demands of the realtime PCR and laboratory equipment is small and can easily be met by small electrical generators, or directly from a vehicle (picture 1). Preservation of reagents in the field by refrigeration is also desirable and a
Picture 1 shows the set up of the real-time PCR workstation in Mbomo, Congo. In the foreground is the thermocycler and its accessories; power is being supplied by means of a 700 watt inverter attached to the battery of the vehicle present. The clinic used in the management of the outbreak is visible in the background, demonstrating the ease at which samples could be collected and handed to laboratory support for testing.

La photo 1 montre l’installation du poste de travail où se réalise la PCR en temps réel à Mbomo, Congo.

Field diagnosis in remote areas such as Mbomo poses many problems for molecular based techniques mainly in the area of power supply and refrigeration. However it was possible to provide timely and accurate testing with a minimum of equipment to provide aid that could be of significant benefit to those affected in the outbreak and those assisting in its management.

Acknowledgements

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Conclusions

Rapid, sensitive and quantitative laboratory diagnosis for Filovirus infections is available in national and international reference laboratories. In this setting, delays and other problems are generally due to improper collection, storage and transport of sensitive samples. In contrast, local laboratory diagnosis has not been standard during EBOV and MARV outbreaks. The Centers for Disease Control and Prevention operated a laboratory during the EBOV outbreak in Gulu (31). The Public Health Agency of Canada provided field laboratory support for an EBOV outbreak investigation in Mbomo, Democratic Republic of the Congo (picture 1).


