IBM Deep Dive Topic 1/14/2015

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Diagnostics and Ebola:

The following is an overview of diagnostic techniques for Ebola infection in humans.

Current Status:

The current status of Ebola diagnostic testing is problematic in West Africa, and has been stated to be so by WHO. The current requirements for cumbersome, slow, and complex diagnostic tests in resource-constrained West Africa has become a major issue and constraint. Existing tests require a dedicated laboratory with a high level of biosafety, expert-level technicians, and expensive and sophisticated machines.

For example, the current 'gold-standard' test, via RT-PCR (Reverse-Transcriptase PCR) requires dedicated primers targeting Ebola viral RNA, a PCR machine, a skilled technician, high BSL precautions, as well as other equipment. The test is labor intensive, requires a full tube of patient blood, takes 2 to 6 hours, and costs approximately $100. This has severely limited testing capacity.

Currently, the WHO is engaged in two diagnostic initiatives regarding the Ebola outbreak in West Africa.

The first initiative is to minimize barriers for new innovations regarding Ebola diagnostic testing, including "clearly defining the needs, by identifying channels to access early validation materials and clinical samples for research and development, and by preparing the deployment of these new tests in the affected countries"

The second initiative is to evaluate the capability and accuracy of available and experimental Ebola diagnostic tests. Currently, Ebola tests have minimal regulatory oversight. No existing rapid Ebola test – whether on the market or in development – have undergone a full regulatory assessment. Thus, research is urgently needed to understand which tests will perform correctly in a field setting.

Signs and Symptoms

In many common viral diseases, diagnostics may only include simple evaluation of signs and symptoms. For example, seasonal influenza is often diagnosed only via signs and symptoms, even though rapid diagnostic tests are available. This is in part due to the fact that diagnosis via signs and symptoms is quite accurate, and the consequences for a false negative or false positive Influenza diagnosis are not particularly catastrophic in most of the population.

Using Influenza as an example, the three symptoms of Fever, Cough, and Nasal Congestion can have (individually) as high as a 98% Sensitivity and 73% Specificity for predictive diagnosis of influenza. This is quite accurate, sensitive, and specific for a test which can be done by a clinician in a matter of minutes by simply evaluating a patient.

Most sensitive symptoms for diagnosing influenza[12]

Symptom:	sensitivity	specificity
Fever	68–86%	25–73%
Cough	84–98%	7–29%
Nasal congestion	68–91%	19–41%

- All three findings, especially fever, were less sensitive in people over 60 years of age.

Such tactics using signs and symptoms which are highly effective for Influenza are simply not viable for Ebola, as the consequence of a false negative in a diagnosis can be catastrophic, while seasonal influenza is generally a self-limiting infection.

Furthermore, Ebola can often have a non-specific and somewhat varied clinical presentation, with symptoms mimicking anything from Influenza to Malaria to Cholera. According to the CDC, a Person Under Investigation (PUI) for suspected Ebola is defined by the following diagnostic criteria:

Person Under Investigation (PUI)

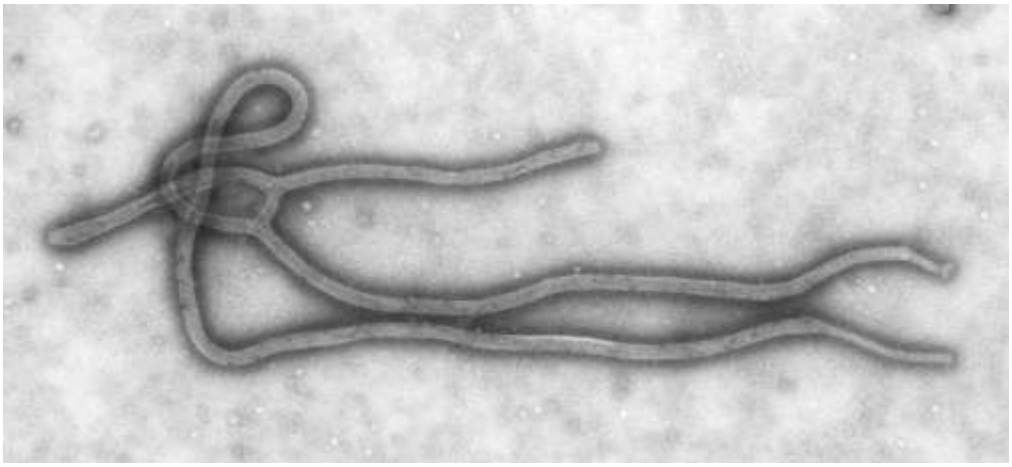
A person who has both consistent signs or symptoms and risk factors as follows:

1. Elevated body temperature or subjective fever or symptoms, including severe headache, fatigue, muscle pain, vomiting, diarrhea, abdominal pain, or unexplained hemorrhage; **AND**
2. An epidemiologic risk factor within the 21 days before the onset of symptoms.

Thus, while signs and symptoms are useful in Ebola infection, they cannot be definitive in terms of their meaning. This is why scientific-based testing is so critically important.

Scientific Diagnostics

Plaque Assay or TEM



When Ebola and other similar hemorrhagic fevers were first discovered and isolated as filoviruses in the 1970s, the definitive identification method was via TEM (transmission electron microscopy). This was a slow, cumbersome process, and can only be conducted far off-site at highly equipped BSL-4 labs in the West. Fortunately, since the 1970s, improvements in biotechnology has made diagnosis via culture assay and TEM a thing of the past.

However, a plaque assay is still often used as an adjunct to validate the results of PCR in clinical and field studies of Ebola. A plaque assay is conducted as follows, and gives results showing the viral load found in a blood sample of a patient.

A plaque assay for a viral infection like Ebola is somewhat analogous in operation and duration to a 'bacterial culture' for a conventional blood infection, but with substantially added complexity and specificity of monoclonal/polyclonal antibody based detection in Vero cell lines. Additionally, a plaque assay for Ebola is time-consuming, expensive, labor-intensive, and requires access to a BSL-4 facility.

A Plaque Assay is rarely used as a diagnostic tool on its own, but rather is used to confirm the results of qPCR, since a viral plaque assay is able to quantify the number of viral plaque-forming units (pfu) per mL of blood (or PBMC) sample.

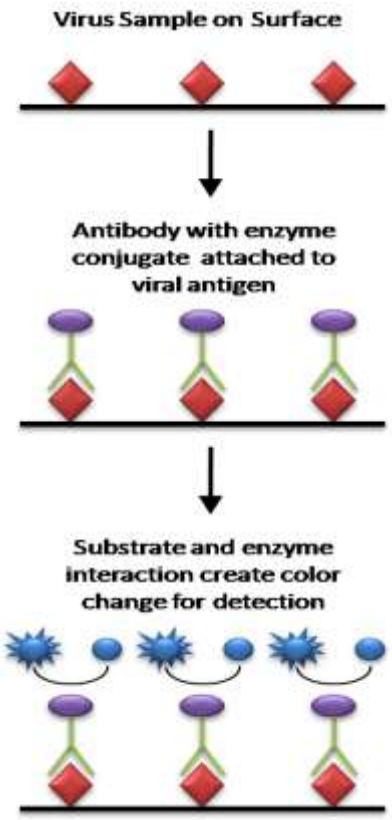
The process for an Ebola Plaque Assay involves incubating the Ebola virus on Vero E6 cells in tissue culture plates on monolayers. After infectious virus is extracted from blood, the resulting purified fluid is added to a serial dilution to the cell monolayers is set up ranging from 10^-1 to 10^-5 dilutions.

The cell monolayers are incubated with appropriate reagents (buffers, agarose, fetal bovine serum, antibiotic, minimum essential medium) for a duration for approximately 7 to 8 days. The plates are removed, rinsed, and decontaminated using Lysol and Gamma radiation.

At this point, the plates are able to be removed from the BSL-4 lab to a lower safety level, where they are exposed to TEM or to an ELISA-type assay. Rabbit-derived antibodies to ebolavirus are used in a 'sandwich ELISA' type procedure. At this point, viral plaques are counted after appropriate staining of the antibodies, and the viral load in the original sample is able to be determined from the serial dilution.

Again, the principal use for this procedure is in academic research rather than clinical practice.

Antibody-Based Assays (Antigen Capture / Sandwich ELISA)



The blood of infected Ebola patient contains (1) Ebola viral antigen (viral protein fragments), (2) fully intact and infectious Ebola virus particles, as well as (3) possibly host immune-derived antibodies to the virus (IgG and IgM). All of these are potential targets for antibody-based assays, but only the former two are used in diagnostic clinical practice.

In the 1999 study by Ksiazek, we learn that:

“Viral antigen (viral protein fragments or live virus) could be detected in virtually all patients during the acute phase of illness, while antibody was not always detectable before death.

IgG and IgM antibody appeared at approximately the same time after disease onset (8-10 days), but IgM persisted for a much shorter period among the surviving convalescent patients. IgG antibody was detectable in surviving patients through about 2 years after onset, the latest time that samples were obtained.”

Detection of Ebola virus antigens or virus isolation *appears to be the most reliable means of diagnosis for patients with suspected acute EHF, since patients with this often-fatal disease (80% mortality) may not develop detectable antibodies before death." (Ksiazek, 1999)*

http://www.ncbi.nlm.nih.gov/pubmed/9988182/

Based on the above knowledge, attempts to detect IgG or IgM anti-Ebola immune antibodies in patients is not a practical or reliable method. Antigen capture (ELISA-derived techniques) are a far more reliable method than looking for antibodies in patients. This is in stark contrast to diagnostic methods for more common and persistent viral infections like HIV, Herpes, and Hepatitis, where most diagnostic tests look for the presence of virus-specific host IgG or IgM antibodies in blood.

Antibody-Based Assays which target Ebola virus antigen incorporate a broad array of diagnostic techniques. Generally, all involve a derivative of ELISA (Enzyme-linked Immunosorbant Assay), often 'sandwich Elisa'. There are varying degrees of automation in ELISA test kits for Ebola.

An example procedure for a traditional ELISA assay for Ebola might look something as follows.

Monoclonal antibodies to Ebola derived from mice are added to suspected infectious fluid derived from patients in a serial dilution. Polyclonal antibodies derived from anti-Ebola rabbit serum are used from antigen detection. Horseradish Peroxidase is used to create a dye-based signal which is detected by a microplate reader to detect signal intensity from a dye linked to enzyme activity (ELISA). http://www.ncbi.nlm.nih.gov/pmc/articles/PMC265191/

Compared to virus isolation and analysis available in the 1970s, even traditional ELISA-based techniques are sensitive and specific (although labor intensive).

More specifically, a modern field technique might work as follows: Antibodies to the virus are bound to a sample and analyzed. An example technique might involve a serial dilution of blood-derived product to 1/100, 1/400, 1/1600, and 1/6400. The optical density (OD) from the serial dilutions is recorded to derive a calibration curve and titers. Sera are considered positive for Ebola if the titer is >=400 and the sum of the adjusted ODs are >0.6.

http://www.ncbi.nlm.nih.gov/pmc/articles/PMC265191/

Given that traditional ELISA-type antibody assays are labor intensive, there are similar rapid diagnostic tests under development for field use which use the same ELISA technology, but in forms suitable for field kits.

Most new 'finger-prick' type field blood tests are based around antibody-based detection. As technology has improved, the level of detection and signal to noise ratios of ELISA based tests have improved as well. Newer Ebola tests which only require 'a single drop of blood' operate in a similar way to home HIV kits, and as are as simple to use as home pregnancy tests.

Unfortunately, early targets of Ebola are in the liver and spleen, not in circulating blood. This limits the utility of any blood-based antibody test to full-blown illness.

Rapid Antibody Test Kits are under development for field use in Ebola. However, when compared to RT-PCR, antibody-based tests are far less reliable, and often to not produce positive results until a patient is well progressed into EVD. The reality is that antibody-based tests are simply not practical in a rapidly-progressing disease such as Ebola.

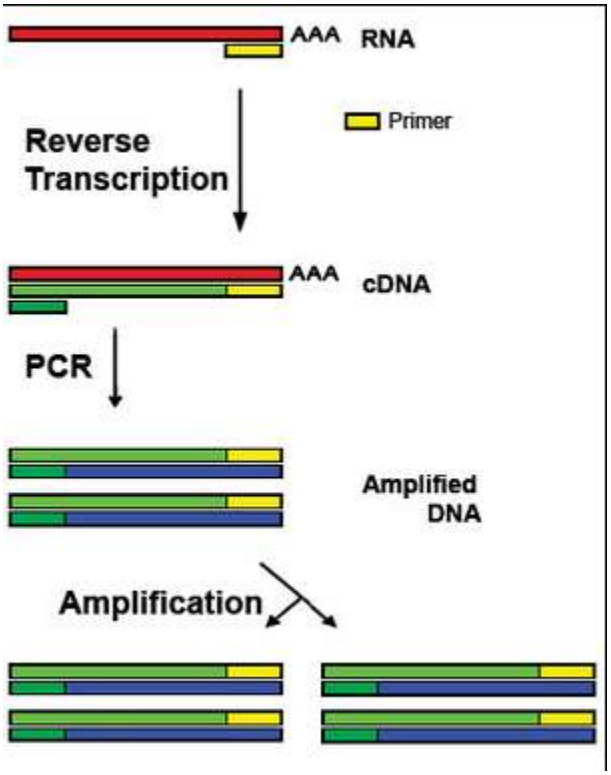
http://www.ncbi.nlm.nih.gov/pmc/articles/PMC265191/pdf/jcm00028-0207.pdf

With a small handful of exceptions, antibody based tests (whether new or old) are simply not as sensitive as PCR based methods.

And since Ebola is not present in blood in substantial concentrations early in infection, this limits the utility of antibody-based tests completely, and leaves us with PCR based methods.

RT-PCR

For diagnostic of viral infection, RT-PCR is currently the 'gold standard' in practice. RT-PCR is reverse-transcriptase PCR. The reverse transcriptase is required to convert Ebola Virus mRNAs or vRNA genome into cDNA transcripts, which then can be amplified using standard PCR with Taq Polymerase using thermo-cycling.



With standard RT-PCR, there is no absolute 'quantification' of viral load, but the result is a binary 'yes/no' answer as to whether targeted Ebola genetic material is present. Once target viral RNA is amplified into cDNA via RT-PCR, various techniques can be used to determine if there is a 'positive' result – in the sense of asking 'Was the target genetic material amplified?'

Once an amplicon is derived from RT-PCR, techniques for detecting the amplicon can include fluorescent oligonucleotide probes or gel electrophoresis, to name two of the more common methods. If the viral amplicon is present after RT-PCR beyond a background cutoff, the patient is considered to be positive for an active Ebola infection. If the amplicon is not present, the patient is likely negative, but may be inconclusive or positive depending on illness phase.

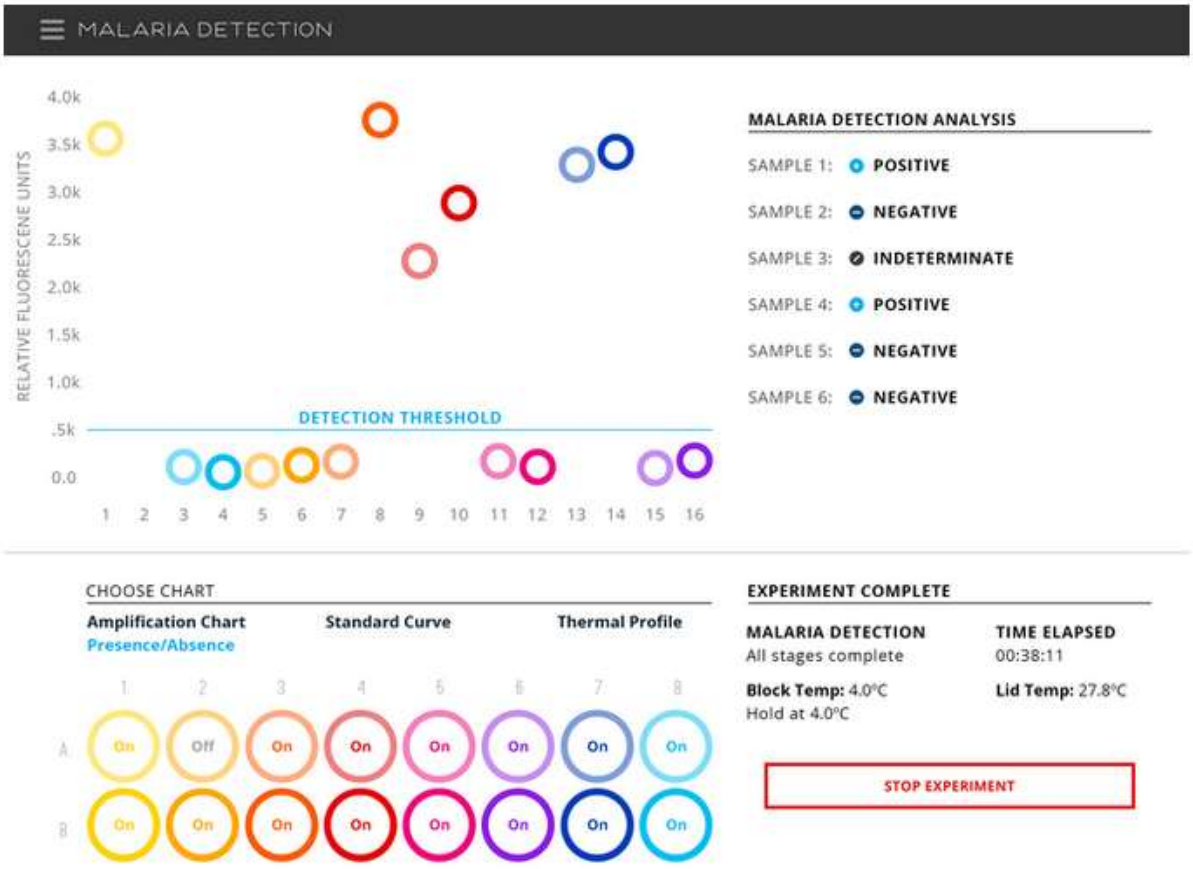
One of the limitations of PCR is that it requires a sufficient amount of viral ssRNA or viral mRNA to be present in the patient's blood. Given that the Ebola virus only infects PBMC in the early stages, the levels of viral RNA may be low to non-existent during early infection (since the virus is in the liver and spleen early in infection, and only moves to PBMC after entering the lymphatic system as vascular integrity becomes more disrupted).

In other words, even RT-PCR has a certain rate (possibly quite high) of false-negatives. In addition to the state or progression of the infection in the patient, RT-PCR is also is somewhat dependent on the primers which are used to amplify a target region of either an mRNA or ssRNA section of the Ebola viral genome. If the primers are not sufficiently complementary to the circulating Ebola strain, false-negatives may result.

For these reasons, it is generally good practice to repeat an RT-PCR test several times over a period of days to ensure a patient is not actually a false-negative. This appears to be common knowledge at field sites in West Africa, where RT-PCR tests are repeated two to three times.

There currently exist both 'conventional' RT-PCR processing kits, which require a skilled technician, as well as 'automated' RT-PCR processing kits, which use a computer and/or robotics to automate much of the workload through specimen processing. Regardless, the underlying process is the same -- convert target viral RNA to cDNA, then detect the amplified cDNA.

Real Time Quantitative RT-PCR (Q-RT-PCR)



Q-RT-PCR is an improvement on RT-PCR. The process is much the same, but Q-RT-PCR is more sensitive and has the ability to actually quantify 'viral load'. This is done through a technique of

detecting changes in fluorescent probes during the process of thermo-cycling, by gathering data on fluorescent excitation and emission of either oligonucleotide dyes or probes. Through this method, one can determine either relative or absolute viral load by looking at cDNA melting curves.

The benefits of Q-RT-PCR is that the process is more than diagnostic -- it can tell a patients viral load, it is more sensitive, and it has a better ability to show the technician if there is an 'inconclusive' result (a patient who may show signs of Ebola cDNA amplification but may be below the background cutoff, thus suggesting that the test be repeated after 24h to 48h).

RT-PCR include conventional RT-PCR diagnostic kits, automated “desktop” PCR systems with integrated specimen processing, and new point-of-care tests that could – within minutes – detect Ebola virus infection with blood from a finger-prick instead of a full tube.

Conclusion:

Currently, sixteen (16) Ebola tests are under evaluation by the WHO as of October 2014. The most promising of these tests are RT-PCR or RT-qPCR based assays which contain a high degree of automation to maximize their utility in the field, and minimize the cost and overhead involved in their deployment because of time and technical limitations in the field.

Regardless, the reality is that due to the pathology of EVD, even the most modern PCR based test cannot diagnose EVD more than 24h prior to the appearance of visible clinical symptoms, according to the CDC. This limits even the best PCR test as a screening mechanism, but does still hold potential for field diagnostics.