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Virological diagnosis of Ebolavirus infection

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Summary

Ebolaviruses, and the other viral causes of haemorrhagic fevers (VHF) have always posed special problems for diagnostic laboratories. These arise from the rarity of human infections, minimal documented experience with test delivery and interpretation, the paucity of established commercial or in-house assays, the lack of clinical material for test development and validation, the high level containment required for handling live virus, the ongoing evolution of the viruses, and the high personal and public health requirements for accurate diagnosis. This article addresses the current situation and the ongoing challenges associated with delivering timely, high quality and safe testing within Australia for people exposed as part of the current major outbreak of Ebolavirus disease (EVD) in Western Africa. The members of the Public Health Laboratory Network have developed deliverable and reliable nucleic acid detection tests, and also have the laboratory capacity to handle the live viruses if necessary. However delivering and maintaining these services necessitates high levels of experience in developing and applying tests for exotic and emerging infections, strong national and international links and collaborations, ongoing monitoring and reassessment of test design and performance, innovative approaches to generation of positive control material, and a regular quality assurance program.

Key words: Diagnosis, Ebola, Filovirus, PCR, quality.

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BACKGROUND

Ebolaviruses, and the other viral causes of haemorrhagic fevers (VHF) with high mortality and the potential for person to person transmission, have always posed special problems for diagnostic laboratories. The current large Ebolavirus disease (EVD) outbreak in West Africa, with cases occurring in returning healthcare workers in a number of developed countries, has raised the level of concern and preparedness in Australia.

The VHF agents are all exotic to Australia and are classed at the highest biosafety level (BSL-4) and require the highest level of physical containment (PC-4). It was recognised in the 1990s that providing the necessary diagnostic support for both suspected and confirmed cases in a timely manner through the small number of PC-4 laboratories nationally was difficult or impossible. In 2002, the Public Health Laboratory Network of Australia pioneered and developed comprehensive guidelines that included a staged approach to testing for patients with suspected VHFs. These have been progressively upgraded[1] and allow laboratories to safely conduct testing, other than cultivation of the virus, within lower level and more widely available PC-3 or PC-2 laboratories. PC-4 level support for VHF-specific tests and for virus culture is available at a national level from the National High Security Quarantine Laboratory (NHSQL) at the Victorian Infectious Diseases Reference Laboratory (VIDRL) in Melbourne (Fig. 1).

The other challenge faced is the intermittent and short-lived nature of previous outbreaks of EVD, which has meant that there has been very limited test development internationally, and even less information on test performance and interpretation. Also, in Australia test development has to be carried out without access to patient material and with tight regulatory restrictions on the importation and distribution of the Ebolaviruses within the country.

This article addresses the current situation and the ongoing challenges associated with delivering timely, high quality and safe testing within Australia for people exposed as part of the current major outbreak of EVD in Western Africa.

Ebolaviruses form a genus within the *Filoviridae* family (Fig. 2). It is enveloped, with a 19 kb single-stranded negative-sense RNA genome,[2] which codes for one non-structural protein (L gene: RNA-dependent RNA polymerase) and six structural proteins: NP gene (major nucleocapsid protein), VP35 (phosphoprotein), VP40 (membrane-associated matrix protein), GP (transmembrane glycoprotein/secreted glycoprotein), VP30 (ribonucleoprotein-associated) and VP24 (membrane associated protein). There are four Ebolavirus species within Africa: Ebolavirus Zaire (EBOV) first found in what is now the Democratic Republic of Congo; Ebolavirus Sudan (SUDV); Ebolavirus Bundibugyo (BDBV) first found in Uganda; and a single case of Ebolavirus Taï Forest (TAFV) infection in the Côte d'Ivoire. Outside Africa, an attenuated species, Ebolavirus Reston (RESTV), was detected in primates from the Philippines, with seroconversion in the primate handlers. The current outbreak of EBOV is due to a strain designated

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Fig. 1 Staff performing Ebolavirus culture under Physical Containment Level 4 conditions at the National High Security Quarantine Laboratory at VIDRL. (Image supplied by Julian Druce.)

as Makona,[3] which appears to have evolved from Central African strains as the virus spread to West Africa in non-human reservoirs over the last decade or more.[4]

DIAGNOSTIC TESTS FOR DETECTION OF EBOV

Virus culture

The filoviruses grow well in Vero and Vero E6 cell lines, although the cytopathic effect may be difficult to detect without passaging.[2] Cell culture is less sensitive than polymerase chain reaction (PCR) and can be performed only in a PC-4 laboratory, so it has very limited use for primary diagnosis. However, it is valuable for providing virus for sequencing (including whole genome sequencing) in order to determine the molecular epidemiology, to monitor changes that may affect the sensitivity and specificity of the nucleic acid detection tests, to guide further test development and to provide positive control material for test evaluation. The last includes the development of improved antigen detection tests and serological assays. Occasionally it may be used for sorting out unexpected positive PCR results, for example in cases occurring outside areas of known activity or where the illness is atypical; and for unusual situations where a false negative PCR is suspected, e.g., patients with a strong clinical and exposure history where the negative result may reflect a genetic variant or a faulty assay.

Viral nucleic acid detection

PCR-based assays have now become both the standard and preferred method for the detection of EBOV virus.[5] Properly performed, they are the most sensitive tests, are highly specific, and can be performed safely in a standard laboratory environment.

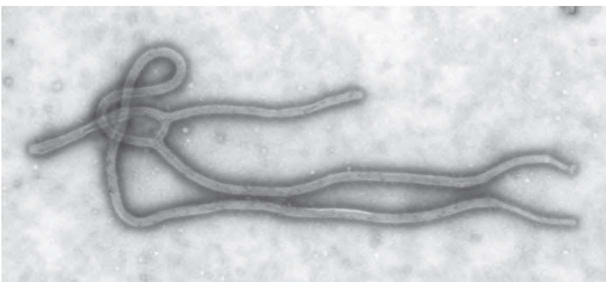

Fig. 2 Transmission electron micrograph of an Ebolavirus virion. (Image accessed from the Centers for Disease Control Public Health Image Library, contributed by Cynthia Goldsmith from the CDC.)

SUDV RNA has been found in a large range of body fluids and tissues in patients with symptomatic diseases, including blood, sweat, saliva, urine, semen and breast milk.[6] Blood is regarded as the single most reliable sample for detection and exclusion of EBOV infection.[5] A study under field conditions from the SUDV outbreak in Uganda in 2001 showed that RNA was present in the blood on the day of onset of illness, rose over the first 5–6 days of clinical illness and peaked around 3.5×10^6 copies/mL in non-fatal cases and 3.5×10^8 copies/mL in fatal cases. However, it did not reach reliable detectable levels (10^5 copies/mL) until 72 h after onset of illness, so that a negative PCR in the first 72 h of illness does not exclude infection.[7] There are anecdotal reports of similar early false negative PCR results in the current EBOV outbreak.[8]

Oral fluid is recommended[5] if a blood sample cannot be obtained. While it was shown to be as sensitive as serum for diagnosis in eight patients in the 2004 Republic of Congo outbreak, data are limited.[9] EBOV has been detected in ocular fluid by PCR and culture for at least 14 weeks,[10] semen by PCR for up to 101 days,[11] and it also has been reported in breast milk in the absence of detection in blood.[12]

Transient low levels of EBOV have been detected by PCR (but not virus isolation) in asymptomatic patients in previous outbreaks,[13] but they have not yet been described in the current outbreak and are not believed to represent an infectious risk.

A number of targets and assays have been used for EBOV detection: those directed against NP, GP and VP40 gene sequences are species-specific and possibly lineage-specific while those directed at L gene sequences can detect all filoviruses.[14] So performance may vary according to the species of the ebolavirus, and not all published assays,[14] including some of those approved by the Food and Drug Administration (FDA) in the United States, have been assessed against the current outbreak strain.[15] For example, the two Centers for Disease Control (CDC) reverse transcription, real-time PCR assays directed at the NP2 gene and the VP40 gene were evaluated using the 1976 Mayinga strain, the 1995 Kikwit strain and the 2002 Gabon strain of EBOV. They performed well, but the sensitivity for the current Makona strain had to be assumed based on primer and probe sequence homology with the evaluated strains.

Ongoing genetic evolution of EBOV within the current West African outbreak may, of course, also affect assay sensitivity and specificity,[4] although there is no evidence of this happening as yet.

Interestingly, a separate small outbreak of EBOV in the Democratic Republic of the Congo in 2014 was due to a different variant of the virus (called Lomela strain), reinforcing the need to be aware of the potential for genetically different strains to emerge.[3]

Provided that an appropriate process is used, filoviruses are inactivated during specimen extraction and lysis,[5,16,17] so that once extraction is performed and the external surfaces are decontaminated, further testing can be carried out at PC-2 level.[1,5] The testing laboratory needs to verify that their extraction process is adequate to inactivate virus[17] and that appropriately high standards of laboratory practice are applied.[1]

Due to the lack of availability of clinical samples for test development, the validation of the PCR assays in use internationally is based almost entirely on the use of live virus,

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