# Viral haemorrhagic fever

## Laboratory case definition

The Public Health Laboratory Network (PHLN) has developed standard case definitions to inform the diagnosis of key diseases in Australia. This document contains the laboratory case definition (LCD) for Viral haemorrhagic fever.

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# 1 PHLN summary laboratory definition

## 1.1. Condition

Acute Viral Haemorrhagic Fevers (VHF) are diseases that may be associated with haemorrhagic syndrome, caused by four distinct families of viruses, arenavirus (Old and New world), bunyaviruses, filoviruses and flaviviruses. Quarantinable VHF viruses include Ebola virus (EBOV), Marburg virus (MARV), Lassa virus (LASV), Lujo virus (LUJV), Crimean-Congo haemorrhagic fever virus (CCHFV), Rift Valley fever virus (RVFV), Machupo virus (MACV), Junin virus (JUNV), Guanarito virus (GTOV), Sabia virus (SABV) and Chapare virus (CHPV).

### 1.1.1. Definitive criteria (one or more of the following criteria)

- Detection of Nucleic Acid
    - Detection of VHF virus-specific RNA in a clinical specimen with one or more RNA targets testing positive and confirmation by nucleic acid sequencing of at least one RNA target.
- Virus isolation
    - Isolation of VHF virus in cell culture from a clinical specimen and confirmed by RT-PCR and nucleic acid sequencing.
- Serological testing
    - A significant rise in virus-specific Immunoglobulin G (IgG) antibody levels (for example four-fold or greater change in titre) between acute and convalescent-phase serum samples.

### 1.1.2. Suggestive criteria (any one or more of the following criteria)

- Detection of VHF virus-specific RNA in clinical specimen with one RNA target.
- Detection of viral antigens in clinical specimen, with one antigen target.
- Detection of VHF virus-specific IgM, with or without detection of virus-specific IgG.

### 1.1.3. Comments

Requests for testing should come endorsed by the Chief Health Officer and/or delegate of the state or territory once satisfied that grounds for testing exist, i.e. following risk assessment (refer to Ebola virus disease – CDNA National Guidelines for Public Health Units Series of National Guidelines (SoNGs)). A single suspected VHF case meeting these criteria is an emergency requiring immediate notification to Commonwealth and jurisdictional public health authorities. For any enquiries or advice about VHF or Human Biosecurity issues please contact your state and territory Health Departments.

# 2 Introduction

## 2.1. Viral haemorrhagic fevers

Viral haemorrhagic fever is a clinical syndrome classically associated with fever and a tendency to bleed. Viral causation of VHF is diverse, involving members of four viral families: *Filoviridae, Bunyaviridae, Arenaviridae* and *Flaviviridae*. This laboratory case definition relates to the subgroup of VHF diseases for which person-to-person transmission potential has been demonstrated together with serious disease, and as such are quarantinable diseases in Australia. The quarantinable VHF are the filoviruses Ebola virus (EBOV) and Marburg virus (MARV), the Old-World arenaviruses Lassa virus and Lujo virus, the New World arenaviruses Machupo virus (MACV), Junin virus (JUNV), Guanarito virus (GTOV), Sabia virus (SABV) and Chapare virus (CHPV) and the bunyaviruses Crimean-Congo haemorrhagic fever virus (CCHFV) and Rift Valley fever virus (RVFV). Infections with Lassa virus are believed to be relatively frequent, occurring year-round in West Africa. The largest VHF outbreak to date was in West Africa caused by EBOV Zaire in 2014-16 (1).

VHF viruses are enveloped RNA viruses. They are maintained in nature in animal hosts, some yet to be definitively identified, spreading zoonotically to humans. The viruses are geographically restricted by the distribution of their natural hosts. Person-to-person spread relies on close contact with infected individuals or their body fluids. Family members and health care providers are characteristically at increased risk of infection. Nosocomial infection has been most marked with EBOV and CCHFV, and is uncommon for Lassa virus. Infections may be severe and life threatening.

## 2.2. Ebola virus

Ebola virus first emerged in 1976 almost simultaneously in the Democratic Republic of Congo (then known as Zaire), and Sudan. EBOV and the closely related MARV are genera within the family *Filoviridae*. The genus *Orthoebolavirus* has 6 species:  EBOV (Zaire strain),

Sudan virus (SUDV), Tai Forest virus (TAFV, formerly Côte d'Ivoire), Bundibugyo virus (BDBV), Bombali virus (BOMV) and Reston virus (RESTV). Reston virus has caused VHF in non-human primates but not in humans. Filoviruses are enveloped, negative-sense, single-stranded RNA viruses. Virions are typically filamentous and of variable length. The RNA genome is approximately 19 kb in length and has the gene order 3′-NP-VP35-VP40-GP-VP30-VP24-L-5′.

The natural reservoir of EBOV remains unknown, but serologic evidence suggests three species of fruit bat in Africa (*Hypsignathus monstrosus, Epomops franqueti* and *Myoncyteris torquate*) and one in the Philippines (*Rousettus amplexicaudatus)* may be reservoir hosts. EBOV ecology is thought to involve maintenance in an enzootic cycle in bat vectors with periodic spillover into non-human primates causing outbreaks with high mortality. Preparation and consumption of bush meat is thought to be associated with spread into the human population. The virus is then transmitted from one human to others via direct contact with blood, secretions, organs or other bodily fluids of infected persons. Traditional burial practices have been identified as a significant risk factor.

EBOV infections and outbreaks have been reported with increasing frequency in various, predominantly central, African countries since 1994, including Gabon, Democratic Republic of Congo, Uganda, Ivory Coast, Republic of Congo, South Africa and Sudan. The mortality rate has ranged from 50-90%. Prior to 2013, the largest outbreak occurred in Gulu in Uganda in 2000/2001 with 425 cases. The 2013-15 outbreak in West Africa was unprecedented in both its West African location and the extent of international spread. This outbreak involved 28,000 known cases with more than 11,000 deaths (2). The outbreak began in Guinea in Gueckedou province near the borders with Liberia and Sierra Leone in December 2013. The outbreak spread to Liberia in March 2014, and to Sierra Leone in May. In March 2014 the Guinean Ministry of Health and WHO were notified of the outbreak, presenting as clusters of fever, severe diarrhoea, vomiting and high mortality (CFR 86%) with deaths in patient family members and healthcare workers. A diagnosis of Ebola virus disease was made by RT-PCR on referred samples by BSL-4 facilities in Lyon and Hamburg. There were ultimately more than 3,000 cases in Guinea, more than 11,000 cases in Liberia, and more than 14,000 cases in Sierra Leone. There was spread within Africa to Nigeria in July 2014 (n = 20 cases), Mali in October 2014 (n = 8) and Senegal in August 2014 (n = 1). Infected nationals, mainly healthcare workers, were repatriated to Spain (n = 2), USA (n = 6), Germany (n = 2), France (n = 2), Norway (n = 1), UK (n = 1), Switzerland (n = 2) and Italy (n = 1). There was also secondary spread to healthcare workers from repatriated cases in the USA (n = 2) and Spain (n = 1). A concerted and prolonged

international effort was required to contain and end the outbreak. More recent EBOV outbreaks include the 2018-2020 Democratic Republic of the Congo outbreak with 3,470 cases caused by Zaire EBOV and the 2023-2023 Uganda outbreak with 164 reported cases caused by the Sudan virus (3).

There have been several documented laboratory accidents involving EBOV, all in research settings. Three involved needlestick injuries to researchers while working with animals (2004 USA, no infection; 2005, Russia, fatal infection; 2009, Germany, survived, treated with VSV/ZEBOV experimental vaccine). A fourth (1976, UK, survived, treated with interferon and convalescent plasma), suffered a needlestick injury while working with a high titre tissue homogenate from an infected animal. The fifth incident, also fatal, occurred in Russia in 1996 from an exposure of undocumented nature. In 2014, a US CDC lab experienced a protocol breach where material from an EBOV experiment, which may have contained live virus, was mistakenly transferred from a BSL-4 lab to a BSL-2 lab. No infection occurred, and the laboratory worker did not develop symptoms during the monitored period of 21 days. Reston virus was first reported in 1989 from several quarantine facilities in Reston, Virginia, USA, where monkeys from the Philippines became ill and died. There have been other reports from facilities in the USA and Italy which also housed monkeys from the same monkey facility in the Philippines. In October 2008, Reston virus infection was confirmed in pigs in the Philippines for the first time (4).

EBOV nucleic acid is typically detectable in the blood for 14 to 21 days. EBOV nucleic acid has been detected in a variety of other body fluids in the absence of blood contamination, including saliva (up to 8 days), rectal swabs (up to 21 days), urine (up to 23 days), conjunctival swabs (up to 22 days), vaginal swabs (up to 33 days), and breast milk (up to 15 days) (5-8). In the wake of the West African outbreak, EBOV was detected in aqueous humour by RT-PCR and culture 14 weeks after clinically resolved acute Ebola virus disease during an episode of pan-uveitis. EBOV shedding has been demonstrated in semen for 12 months or more in 5% of a prospectively followed cohort of Ebola virus disease survivors; the lengthiest documented shedding to date being 565 days (9).

## 2.3. Marburg virus

Marburg virus, and the closely related Ebola virus are genera within the family *Filoviridae*. The genus *Orthomarburgvirus* has 2 species: Marburg virus (MARV) and Ravn virus (RAVV). Virions are typically filamentous and of variable length. The RNA genome is approximately 19 kb in length and has the gene order 3′-NP-VP35-VP40-GP-VP30-VP24-L-5′. Marburg virus was first recognised in 1967 when simultaneous outbreaks of

haemorrhagic fever occurred involving the former Behringwerke AG in Marburg and the Paul Ehrlich Institute in Frankfurt, Germany, and the Institute for Immunology and Virology in Belgrade in the former Yugoslavia. A total of 32 people were infected, of whom 31 were hospitalised, and 7 died (case fatality rate 22%). Those first affected had been exposed to blood, organs and cell cultures from African green monkeys imported from Uganda for work related to *poliovirus* vaccines. There were six secondary cases arising from needle stick injuries (n = 3); sexual intercourse (n = 1); knife cut at post-mortem (n = 1); as well as one case of nosocomial transmission. Marburg viruses are endemic to Africa. The African fruit bat *Rousettus aegyptiacus* are a primary reservoir host, but there may be other host species.

Outbreaks have subsequently been reported in the Democratic Republic of Congo (1998-2000, 154 cases, 128 fatal, case fatality rate 83%), Angola (2004-5 252 cases, 227 fatal, case fatality rate 88%) and Uganda (2012, 26 cases, 15 fatal, case fatality rate 58%), predominantly among mine workers (10). In 2008, two cases (one death) were reported in tourists, one Dutch and the other American, returning from Uganda. Both travellers had visited Kitum cave inhabited by fruit bats in a national park. In 2023, Equatorial Guinea reported the first Marburg Virus Disease (MVD) outbreak (40 confirmed and probable cases, 12 deaths, case fatality rate 30%). In the same year, Tanzania reported nine cases and six deaths (case fatality rate 67%).

Most recently in September 2024, Rwanda reported its first MVD outbreak, with 66 cases and 15 deaths (case fatality rate 23%). Many of those affected were health care workers who worked in intensive care units. During this outbreak, 75% of patients recovered. However, recovery from MVD can be slow and known sequelae include orchitis, recurrent hepatitis, transverse myelitis and uveitis. Of note, Marburg virus can remain detectable in the semen of some patients who recovered up to 203 days after the onset of disease, however there is no strong evidence for sexual transmission of the virus (11). There have been no new cases since October 30, 2024 (12).

Two known laboratory accidents have occurred in the Scientific-Production Association ‘Vector’ laboratory Novosibirsk, Soviet Union in 1988 and 1990, one fatal, with a secondary case thought to have occurred in an investigating pathologist.

## 2.4. Crimean-Congo haemorrhagic fever virus

Crimean-Congo haemorrhagic fever virus (CCHFV) is a member of the genus *Orthonairovirus*, family *Nairoviridae*. Orthonairoviruses are enveloped, RNA viruses, approximately 80-120 nm in diameter. The single-stranded, negative-sense RNA genome is segmented with short (S), medium (M) and long (L) genome segments totalling 17.2-20.1 kb.

Genome segments encode the nucleocapsid protein (N), glycoprotein (GP) and the large (L) protein. The viral genome is highly variable between CCHFV isolates, but the deduced amino acid difference in the N is only 8%, and isolates are regarded as representing a single serotype.

CCHFV, like all nairoviruses is tick-borne and is distributed over the geographic range of ixodid ticks, genus *Hyalomma*, including Africa, Eastern Europe, the Middle East and the west of China. Infection occurs through tick bites, or by direct contact with infected animals or humans or their tissues. Herbivores such as cows, sheep, goats have been implicated in transmission, while birds, although not susceptible to disease, may host and disseminate ticks. In temperate climates, peak transmission generally occurs in the spring and summer during peak population of ticks and their vertebrate hosts. The infection risk from infected ticks is ill-defined but thought to be high.

CCHF in humans is a severe illness with high mortality, but fortunately cases occur infrequently. Most cases occur in the livestock industry including agricultural workers, slaughterhouse workers and vets. However, CCHFV has repeatedly caused nosocomial outbreaks with high mortality rates among healthcare workers, and laboratory workers. Percutaneous exposure represents the highest transmission risk and tertiary cases amongst family members of healthcare workers are well described.

CCHFV is endemic in parts of Africa, Asia, the Middle East and Eastern Europe. In Africa, outbreaks have been reported from South Africa, Congo, Mauritania, Burkina Faso, Tanzania and Senegal. An outbreak in Western China in 1965 had a case fatality rate of 80%. A large number of cases have also been reported from Middle Eastern countries such as Iraq, United Arab Emirates, Saudi Arabia and Oman. Since 2000, outbreaks have been reported in Albania, Kosovo, Turkey, Pakistan, Iran, Mauritania and Kenya. Recent data from South Africa reported 3 cases in 2009 (case fatality rate 33%), compared with 11 cases in 2008 and a single case in 2007. Afghanistan has experienced an increase in numbers with 1,236 CCHF cases reported in 2023 (13).

In Europe, CCHFV is currently endemic only in Bulgaria where a total of 1,568 CCHF cases were notified from 1953 to 2008, with an overall case fatality rate of 17%. However, there has been an increase in cases and outbreaks of CCHFV recorded in other countries in the region such as Albania, Kosovo, Turkey and the Ukraine as well as south-western regions of the Russian Federation and Greece. Since 2020, cases of CCHF have been reported in Spain and Greece, and continue to be reported in Bulgaria (14). This increase has been

attributed to climate and anthropogenic factors such as changes in land use, agricultural practices and movement of livestock, potentially influencing tick-host dynamics.

## 2.5. Lassa virus

Lassa virus is an arenavirus (Family *Arenaviridae*, genus *Mammarenavirus*). Arenaviruses are enveloped and pleomorphic, including round and oval particles ranging in size from 40-200 nm. The genome is bi-segmented, negative-sense, single-stranded RNA, divided into L segment (approximately 7.2kb) and S segment (approximately 3.5kb in length).

The mammalian hosts of arenaviruses are rodents, in the case of Lassa virus the natural hosts are *Mastomys* species (multimammate mice). Rodent hosts may shed virus into the environment via urine or faeces and vertical infection maintains continuity of infection in the host population. Human infection occurs through the inhalation of particles contaminated with rodent urine or saliva or direct contact of broken skin with rodent faeces. Human to human transmission can occur via contact with infected persons bodily fluids such as blood, saliva, urine, or semen. *Mastomys* are common in houses as well as savannah and forests in west, central and eastern Africa. Human infection presumably occurs in and near houses, including when rodents are sought for food. Since the first outbreak of Lassa virus in 1969 in Nigeria, extensive human infection, including occasional nosocomial outbreaks have been reported from Nigeria, Liberia, Sierra Leone, and Guinea. More recently, 6,732 suspected and 1,181 confirmed cases were reported in Nigeria, with 224 deaths in 2020 (15). In 2023, the deadliest outbreak in decades recorded 9,155 suspected cases, 1,270 cases were confirmed, with 227 deaths (15, 16). Serological studies and clinical reports show that Lassa is endemic across West Africa between Nigeria and Senegal. It is thought that Lassa is relatively common, causing tens to hundreds of thousands of human infections annually, and hundreds to thousands of deaths.

Endemic Lassa virus transmission occurs year-round with an increase in case numbers during the dry season (January to April), potentially influenced by increased viral aerosol stability in lower relative humidity, or the seasonal dynamics and behaviours of the *Mastomys* host. Most nosocomial outbreaks occur during the dry season. Percutaneous exposure, contact with infected body fluids and aerosols generated by patients have all been implicated as causes of nosocomial infection.

## 2.6. Lujo virus

Lujo haemorrhagic fever (LUHF) is a disease caused by the Lujo virus, a rare and highly pathogenic Old-World arenavirus first identified in 2008. Its name is derived from "Lusaka" (Zambia) and "Johannesburg" (South Africa), the locations associated with the initial outbreak. The virus was discovered following a nosocomial (hospital-acquired) outbreak involving five people, of whom four died, highlighting its high case fatality rate (~80%). The natural reservoir of Lujo virus is believed to be rodents, particularly species of the *Mastomys* genus (multimammate rats), which are also reservoirs for other arenaviruses like Lassa virus. Human infection with Lujo virus is likely through contact with rodent excreta, urine or saliva. Human-to-human transmission occurs via direct contact with infected bodily fluids, especially in healthcare settings.

## 2.7. Rift Valley fever virus

Rift Valley fever (RVF) is a zoonotic disease caused by Rift Valley fever virus (RVFV), a member of the *Phenuiviridae* family, genus *Phlebovirus*. RVFV is a negative-sense, single-stranded RNA virus with a tri-segmented genome consisting of large (L), medium (M), and small (S) RNA segments. The virus primarily infects livestock (e.g. cattle, sheep, goats) but can also cause severe illness in humans. In humans, RVF mostly manifests as a self-limiting febrile illness but occasionally progresses to severe disease including haemorrhagic fever, encephalitis causing up to 3% mortality. RVFV is primarily transmitted through mosquito (genus *Aedes* and *Culex)* bites, but it can also spread through contact with infected animal blood, tissues or bodily fluids. There have been no reports of person to person spread. RVFV was first identified in 1931 in Kenya, in the Rift Valley region. Since then, 67 RVF outbreaks have been documented across East African countries including Uganda, Rwanda, Kenya, Tanzania, Burundi and South Sudan with confirmed cases and fatalities (17). Periodic outbreaks in other parts of Africa, the Arabian Peninsula, and in parts of Asia. Outbreaks of RVF typically occur following heavy rains or flooding, which increase mosquito populations and create favourable conditions for transmission.

## 2.7 New World arenaviruses

New World arenaviruses are a group of viruses endemic to the Americas and are known for causing viral hemorrhagic fevers in humans. These are Junin, Machupo, Guanarito, Chapare and Sabiá viruses, the cause of Argentine, Bolivian, Venezuelan, Chapare and Brazilian haemorrhagic fever, respectively. These viruses are transmitted primarily through contact with infected rodent faeces.

# 3 Tests

In Australia, the safety and quality of VHF diagnostic testing are best ensured by restricting testing to Public Health Laboratory Network (PHLN) member laboratories, which are equipped with high-quality facilities, trained personnel, and the capability to accurately assess and effectively manage biosafety risks.

The National High Security Quarantine Laboratory (NHSQL) at the Victorian Infectious Diseases Reference Laboratory (VIDRL) serves as Australia's designated national reference laboratory for quarantinable VHFs. VIDRL provides diagnostic capability and confirmatory testing on an urgent, on-call basis, 24 hours a day, 7 days a week.

## 3.1. Access to diagnostic testing

Access to testing by expert PHLN member laboratories is available in some jurisdictions. This can be accessed via their respective State or Territory Chief Quarantine Officer.

NHSQL at VIDRL is available to provide primary, parallel and confirmatory testing for all jurisdictions, as required. All positive tests obtained at a jurisdictional level must be confirmed at NHSQL.

Direct contact with the medical microbiologist on-call at VIDRL is essential to arrange receipt of specimens and obtain advice on specimen collection, safe packaging and transport.

The VIDRL on-call medical microbiologist can be contacted on (03) 9342 9600 during business hours Monday to Friday, or via the Royal Melbourne Hospital switchboard on (03) 9342 7000 after hours.

Polymerase chain reaction (PCR) is the primary diagnostic modality employed for detection of VHFs by the NHSQL, and collection of appropriate specimens for this purpose is a priority.

### 3.1.1. Specimen collection

The essential specimens to be submitted for virus detection are:

1. Whole blood in an ethylenediaminetetraacetic acid (EDTA, usually mauve-top) tube.
2. Oral fluid or swabs and urine only if blood collection is not possible (e.g. in the post-mortem setting). Ocular samples and semen may be collected.
3. If post-mortem specimens are available, serum, liver, lung, spleen and kidney tissues are desirable.

Appropriate equipment includes:

- Personal protective equipment (PPE). For more information see EVD SoNG and Infection Prevention and Control Expert Advisory Group (IPCEAG) report[1].
- Screw-capped (O ring sealed) plastic tubes containing viral transport medium
- A fine tip permanent marker pen
- Waterproof plasters
- A sealable plastic specimen bag. Absorbent packaging material and a strong outer container and 0.1% hypochlorite solution to wipe-down the outside of the primary container before transport to the laboratory
- 'High Risk' labels
- A clinical waste bag for disposal of discarded dressings and personal protective equipment.

The following procedures should be followed:

- Venous blood samples must be collected with extreme care to avoid self-inoculation. Needles should not be recapped, bent, broken, removed from disposable syringes or otherwise handled.
- For adults collect ten millilitres, and for children collect minimum of 1 millilitres blood anti-coagulated with EDTA (NOT heparin) and place in a sealed plastic container.
- Do not separate or remove serum or plasma from the primary collection container.
- Blood-taking equipment should be placed into a puncture-proof approved sharps container. When full, the container should be placed in a plastic bag, sealed and the outside wiped over with 0.5% hypochlorite, marked with the nature of the contents, and then autoclaved or incinerated.
- Oral swabs should be placed in plastic screw-cap containers of 1 mL of sterile, viral transport medium (Minimum Essential Medium plus 2% foetal calf serum, penicillin 100 units/mL, streptomycin, 100 ug/mL neomycin 40 ug/mL and amphotericin B 20 ug/mL; available from VIDRL on request) or equivalent viral transport medium (VTM). A dry swab should be collected if no VTM is available.

### 3.1.2. Transport of specimens from primary laboratory to the NHSQL at VIDRL.

[1] Infection Prevention and Control Expert Advisory Group (IPCEAG). Infection prevention and control principles and recommendations for Ebola virus disease - Including information about personal protective equipment for clinical care of patients with suspected or confirmed Ebola virus disease in the Australian healthcare setting. Canberra (ACT): Commonwealth of Australia 2015; 2015. Report No.: 1105.

The outside of each specimen container should be wiped with disinfectant (0.1% hypochlorite solution) and a label should be attached bearing the patient's name, hospital identification, the date of collection and the nature of the suspected infection. The specimens should be double bagged in secure, leakproof bags, which have been similarly labelled. Bags containing specimens should be wiped with disinfectant before being removed from the patient's room.

Samples should be identified as: "Infectious Substances, Affecting Humans (Ebola virus or Marburg virus)" and packaged and handled as required by the International Air Transport Association (IATA) packing instruction 620.

The specimens should be packaged as follows:

**Primary Container**

Place the specimens for transport in securely sealed, watertight container, such as a screw-cap plastic tube or vial. Ensure plastic containers are resistant to temperatures as low as -80°C.

**Secondary Container**

Wrap the primary container with sufficient absorbent material to absorb the entire contents from any leaks or spills if the primary container fails. Place the wrapped, sealed primary container into a durable, watertight, screw-lid plastic container (secondary container).

Several primary containers (individually wrapped with absorbent and cushioning material to prevent breakage of fragile samples) may be placed in one secondary container to a maximum of 50 mL of specimen material. On the outside of the secondary container, attach the specimen labels and other relevant information.

**Outer Shipping Container** This container must be rigid and certified for shipping Category A infectious substances. Place the secondary container inside the outer shipping package and addressed to:

**National High Security Quarantine Laboratory**
Victorian Infectious Diseases Reference Laboratory
The Doherty Institute
792 Elizabeth Street
Melbourne VIC 3000

Only use an approved courier for the transport of Category A infectious substances. Because individual commercial and non-commercial carriers or shipping services may apply different regulations for transporting biological specimens, contact a representative of the chosen carrier beforehand to ensure that all necessary formalities are fulfilled.

Notify the on-call VIDRL medical microbiologist of the dispatch of the specimen and flight time and number, courier or airway bill number as appropriate. If transport is by air, a dangerous goods declaration must be made (refer to the IATA Dangerous Goods regulations).

#### 3.1.3. Specimen Handling

Clinical samples from suspected cases must be handled with due regard to the likelihood that VHF viruses may be present, and the appropriate procedures observed. PC4 containment is mandatory for virus isolation in cell culture. Should it be necessary to conduct work other than in a PC4 laboratory, a full risk assessment must be conducted. Specific diagnosis of VHF viruses by reverse transcriptase PCR (RT-PCR), antigen detection (in the unlikely event that it is attempted) or detection of VHF virus-specific antibody may all be done by sufficiently expert PHLN member laboratories with appropriate facilities and equipment, expert staff and robust standard operating procedures. This should be employing methodology for managing the infectious risk posed by specimens consistent with separate detailed PHLN guidelines for nucleic acid testing and other clinical pathology for infectious agents performed on specimens from VHF patients.

These guidelines are available on the PHLN Laboratory Case Definition website. Note that there is published evidence of some variability in effectiveness of proprietary nucleic acid extraction buffers in inactivating filoviruses, and bear in mind the high viral titres that may be present in specimens.

# 4 Laboratory Testing

## 4.1. The filoviruses Ebola virus and Marburg virus

### 4.1.1. Samples

Serum or plasma is the diagnostic sample of choice for the laboratory diagnosis of EBOV and MARV disease. A relatively high titre viraemia is typically present throughout the acute clinical illness. Extensive published literature is available documenting the use of serum or plasma for direct detection of virus by RT-PCR, antigen detection, virus isolation in cell culture, electron microscopy, and immune markers.

Although drawing and handling of blood specimens is highly standardised in clinical and laboratory practice in developed countries there are some drawbacks, mainly under field conditions. Availability and safe handling of phlebotomy equipment and cultural resistance to blood sampling can present challenges. There is some data to suggest that oral fluid can be

used for the detection of EBOV (7) and MARV (18). Therefore, this is a worthwhile specimen to collect only if phlebotomy is not possible, as a supplementary specimen to increase robustness of diagnosis, and in the post-mortem setting.

Male EBOV survivors should be offered semen testing at 3 months after onset of disease, and then, for those who test positive, every month thereafter until their semen tests negative for virus twice by RT-PCR, with an interval of one week between tests (19).

### 4.1.2. Nucleic acid testing

Nucleic acid testing, performed primarily on serum or plasma, has become the preferred diagnostic method for EBOV and MARV Samples will be inactivated in a PC4 laboratory prior to molecular processing at PC2.

A variety of in-house conventional RT-PCR and real-time RT-PCR assays have been developed targeting the Nucleoprotein (NP), Glycoprotein (GP), or occasionally L gene for EBOV detection and the VP35 and L gene for MARV detection. PCRs have been shown to be sensitive and specific, including under field conditions in the laboratory diagnosis of EBOV. Based on *in-silico* analysis for PCR primer mismatches against the 2024 Rwandan MARV strain, it is expected that analytical sensitivities of MARV PCR assays remain unchanged. Some commercial assays for MARV detection are available. These include, but are not limited to, the FilmArray BioThreat Panel (BioFire), the FilmArray Global Fever Panel – Research Use Only (BioFire), RealStar Filovirus Type RT-PCR Kit 2.0 (altona DIAGNOSTICS) and RealStar Filovirus Screen RT-PCR Kit 1.0 (altona DIAGNOSTICS).

Viraemia can be reliably detected from the time of presentation for at least 14 days; occasionally for as long as 21 days. To prevent false negatives, results obtained in the first 48 hours of illness should be confirmed by a second specimen obtained after this time when a high degree of clinical suspicion exists. Viral load (by RT-qPCR) appears to correlate with clinical outcome, with viral load of >6 log10 correlating with lower survival (20).

False negative PCR results had been reported in VHF during acute phase (low viraemia) or presence of inhibitors in clinical samples (21). Inhibition controls are important with RT-PCR assays to guard against false negatives.

To control for PCR inhibition in case of EBOV or other VHFs detection, an exogenous internal control like Bovine Viral Diarrhea Virus (BVDV), is added before RNA extraction. This control helps evaluate the yield of spiked RNA and indicates if the testing is valid before analysing the EBOV or other VHF target sequence.

### 4.1.3. Virus isolation

Virus isolation from serum or other clinical material remains a reference method for confirmatory testing, reagent production, test validation or research. Liver is the most suitable source of virus after serum, while throat washings or urine samples have less commonly yielded virus. PC4 containment is required for virus amplification in cell culture. Commonly used cell lines include Vero E6 (ATCC CRL-1586) and MA-104 (ATCC CRL-2378.1) cells, although filoviruses will grow in a range of cell lines. Cell culture is relatively sensitive, although cytopathic effect can be variable. Viral replication can be monitored by microscopy, immunofluorescence or RT-qPCR.

The filoviruses EBOV and MARV are Tier 1 Security Sensitive Biological Agents (SSBA) in Australia. They may only be stored or handled in an appropriately accredited facility by authorised staff like NHSQL-VIDRL.

### 4.1.4. Antigen detection

Detection of filovirus antigens, most commonly using monoclonal antibodies in an antigen-capture EIA has been widely used in the laboratory and in the field. The target proteins are typically NP, VP40 and GP. Antigenaemia has been shown to clear with the appearance of IgM on average 1-2 days before RT-PCR became negative. Head-to-head comparison of four Rapid Antigen Tests (RATs) (22) with EBOV GeneXpert on whole blood collections demonstrated poor sensitivity (range 21.6-61.6%) but high specificity (97.5-99.1%) for RATs, hence RATs are only recommended for use in very restricted setting in which RT-PCR is not readily available (23).

### 4.1.5. Antibody detection

Filovirus serology can be useful for seroepidemiological purposes and as an adjunct to direct virus detection, especially in survivors during the convalescent period. Its utility in acute diagnosis is limited however, by the frequent failure for an antibody response to be mounted in fatal cases, which given high filovirus mortality rates is a significant proportion of cases.

Indirect immunofluorescence with native virus antigens produced in Vero cells has been used in IgM and IgG detection over a long period of time. Some variability in sensitivity and specificity has been noted. Native virus antigen IgG enzyme immunoassay (EIA) and IgM capture EIA have been produced using gamma-irradiated cell culture grown antigens. These assays have been used on human outbreak derived samples to show that IgM and IgG antibodies appeared 8-10 days after illness onset. IgG persisted in survivors for as long as two years. In non-human primates IgM was detected on day 6 of infection and persisted for

at least 84 days. IgG was detected on day 10-12 and persisted for more than 400 days. A significant disadvantage with native antigen assays is the need for PC4 facilities for antigen preparation, and the limits this places on availability and standardisation.

Recombinant filovirus antigens including VP40, NP, VP35, GP or VP30 have been produced for serologic assays. Assays based on NP of both EBOV and to a lesser extent MARV have been evaluated using human and non-human primate sera. Assays based on GP and VP35 antigens have been developed (24). Commercial EIA reagents now exist but may not be available in Australia.

Serology tests are a critical diagnostic tool for detecting VHFs, as they can identify the presence of virus-specific antibodies (IgM and IgG) or antigens in a patient's blood. These tests help confirm infection, particularly in areas where VHFs are endemic or during outbreaks. The primary methods include enzyme-linked immunosorbent assays (ELISA), which detect and quantify antibodies or viral antigens, and indirect immunofluorescence assays (IFA) for visualizing specific antibody responses. In addition, virus neutralization assays (VNAs) are considered the gold standard for assessing the functional capacity of antibodies to inhibit viral replication. Detection of IgM antibodies typically indicates recent infection, and the antibody titre can decrease after one month, while IgG antibodies can signify past exposure or immune response to vaccination (25). Apart from clinical diagnosis, serology is used for epidemiological surveillance of EBOV infections before and after outbreaks, and to potentially assess the impact of EBOV vaccination effort (26).

Despite their utility, serology tests for VHFs have limitations. Antibody responses may not be detectable in the early stages of infection, including in non-survivors (24). Although species-specific assays using secreted glycoproteins have been developed, cross-reactivity among antibodies (IgM>IgG) within a genus have been seen. Virus neutralisation assays require live virus and containment facilities, limiting their use in resource-constrained settings. Advances in rapid diagnostic test (RDT) technology and point-of-care assays are improving the accessibility and speed of serological testing, while VNAs remain essential for research, vaccine evaluation, and detailed immune response studies.

### 4.1.6. Electron microscopy

Modern advancements in transmission electron microscopy (TEM) have significantly enhanced its utility for detecting EBOV, in suitably equipped facilities. High titres of the virus are often present in serum and plasma, enabling direct visualisation via negative staining and TEM examination of patient samples. Additionally, rapid thin section protocols tailored to the identification of viral particles within tissues or infected cell culture materials, are also of

diagnostic value. While nucleic acid testing remains the primary diagnostic method due to speed and specificity, TEM serves as a crucial supplementary tool when molecular assays are inconclusive or unavailable for the specific agent in question. As a gold standard for virus characterisation, TEM continues to play an important role alongside other routine diagnostic methods.

#### 4.1.7. Sequencing

Sanger sequencing of amplicons generated by conventional RT-PCR can be used to confirm the specific detection of EBOV or MARV. This includes partial NP gene sequence and partial L gene sequence for EBOV, and partial VP35 gene sequence and partial L gene sequence for MARV.

Whole genome sequencing using next generation sequencing technologies (e.g. Illumina and Oxford Nanopore) can be utilised to confirm the molecular detection of filoviruses, and support contact tracing efforts through detailed phylogenetics and genomic epidemiological analysis. Unlike traditional molecular diagnostic assays, whole genome sequencing using shotgun or pan-viral hybridization/capture metagenomic approaches is highly tolerable to viral genome diversity and evolution, providing a useful tool for monitoring molecular diagnostic assay escape during an outbreak (27-29).

### 4.2. Crimean-Congo haemorrhagic fever virus

#### 4.2.1. Samples

Serum or plasma is the diagnostic sample most commonly used for nucleic acid testing, virus isolation, antigen detection or serology. Detection has also been achieved in saliva and urine. Post-mortem when blood is not available tissue suspensions made from liver needle biopsies may be of utility for viral studies.

#### 4.2.2. Nucleic acid testing

As for other quarantinable VHF, nucleic acid testing has become the mainstay of diagnosis for CCHFV. RT-PCR is the method most commonly employed. Both conventional and real-time assays have been successfully used. The diversity of viral strains is a significant potential challenge in designing PCR primers and probes. The NP is the most frequently employed target as it is the most conserved gene. Viraemia can generally be detected for up to 18 days post disease onset. Quantitative methods allow assessment of virus load which several reports suggest has prognostic implications. Loads greater than $10^8$ copies/ml in plasma can be considered predictive of fatal outcome.

### 4.2.3. Viral isolation

Virus isolation remains a reference method, but has been surpassed in sensitivity, speed and convenience for diagnostic purposes by nucleic acid testing. PC4 containment is required for virus amplification in cell culture for confirmatory testing, reagent production, test validation or research. CCHFV replicates in a variety of cell lines with variable CPE. After 1-6 days of cell culture, confirmation of replication by IFA or RT-PCR is usually required due to lack of CPE. Virus isolation is generally only possible when relatively high levels of viraemia are present during the first 6 days of illness, although positive isolations can sometimes be made until day 12. Viraemia levels detected by culture peak at log10 3.7 Focus-forming units with a mean of log10 3.4 Focus-forming units.

### 4.2.4. Antigen detection

Antigen detection by capture EIA or reverse passive haemagglutination tests are largely supplanted by RT-PCR which significantly exceeds their sensitivity. The NP is immunodominant and is usually targeted. Antigen may be detected in serum or plasma in the first 11 days of illness, more commonly in fatal cases.

### 4.2.5. Antibody detection

Detection of IgM and IgG may be accomplished using EIA or IFA formats. Commercial reagents exist but may not be available in Australia. Complement fixation, gel diffusion, haemagglutination inhibition (HAI) and other test formats have been used in the past but have limited sensitivity compared to newer EIA and IFA. Antibody is not detectable in the first few days of illness. In non-fatal infections EIA and IFA can detect IgM and IgG antibody reliably from day 5, although EIA may sometimes detect antibody as early as day 3. IgM responses decline from 2-3 months after infection to be undetectable at 4-6 months. IgG may remain demonstrable for at least 5 years. Fatal cases usually mount no, or low titre, antibody response. Specificities of IgM and IgG serology by EIA & IFA have been shown to approach 100%. Sensitivity of IgM and IgG detection by IFA (94% and 86%) have exceeded those achieved by EIA (88% and 80%).

### 4.2.6. Whole genome sequencing

Whole genome sequencing using next generation sequencing technologies (e.g. Illumina and Oxford Nanopore) can be utilised to confirm the molecular detection of CCHFV, and support contact tracing efforts through detailed phylogenetics and genomic epidemiological analysis. Unlike traditional molecular diagnostic assays, whole genome sequencing using shotgun or pan-viral hybridization/capture metagenomic approaches is highly tolerable to

viral genome diversity and evolution, providing a useful tool for monitoring molecular diagnostic assay escape during an outbreak (27-29).

## 4.3. Lassa Virus

### 4.3.1. Samples

Serum or plasma is the most commonly used diagnostic sample for nucleic acid testing, virus isolation, antigen detection or serology. Virus may also be recovered from CSF, throat washings, pleural fluid or urine.

### 4.3.2. Nucleic acid testing

RT-PCR has become the diagnostic method of choice for diagnosis of Lassa fever. The diversity of Lassa virus strains makes design of reliable real time PCR probes extremely challenging, however, conventional RT-PCR formats continue to be used for Lassa virus detection in many laboratories for this reason. Amplicon sequencing, nested primer sets, or low-density microarray hybridization may be coupled with RT-PCR to achieve specific detection of a range of Lassa virus strains. In a diagnostic context increased turnaround time, and risk of contamination are inherent disadvantages compared to the rapidity and closed tube format of real time RT-PCR. Real time RT-PCR using Syber Green intercalation is still possible for quantitative purposes if this is desired. The Lassa virus S gene segment coding GPC and NP proteins is the target most commonly used for PCR primer design. Exploration of alternative targets that might offer greater conservation across diverse Lassa virus strains is currently being done with some success.

Lassa virus RNA may be detected in blood with a relatively high degree of reliability between day 3 and day 9 of illness, peaking on day 6. In some patients RNA may be detected out to day 21. Viraemia of between $10^6$ and $10^9$ RNA copies/mL have been described. PCR inhibition due to substances present in blood samples has been described for patients infected with Lassa virus, EBOV and yellow fever virus. Inhibition controls should be included in PCR assays to guard against false negatives.

### 4.3.3. Viral isolation

Virus isolation remains a reference method for the detection of Lassa virus and may be achieved from serum, CSF, throat washings, pleural fluid or urine. Cytopathic effect may be variable but is evident in Vero E6 cells. PC4 containment is required for virus amplification in cell culture which may be undertaken for confirmatory testing, reagent production, test validation or research. Clinical sensitivity is slightly lower than RT-PCR declining steadily from day 3.

#### 4.3.4. Antigen detection

Antigen detection can be done using an EIA format and performed on inactivated specimens with applicability in field conditions. However, clinical sensitivity is approximately a third of RT-PCR, and antigen detection is largely limited to the first 7 days of illness before appearance of IgM.

#### 4.3.5. Antibody detection

Serology is not the method of choice for acute diagnosis of Lassa fever. Specific IgM antibody begins to be reliably present in the second week of illness, prior to which it may be detected in approximately half of patients. Patients with fatal Lassa fever produce relatively low antibody titres or may not develop antibodies at all. Seropositivity increases during the course of disease and reaches high levels by day 18 when viraemia is already decreasing.

Indirect IFA using virus-infected cells as antigen has been the most common serological test method. A four-fold increase in IgG titre or detection of IgM together with IgG has been considered evidence of infection.

EIA and immunoblot tests using recombinant antigens (NP, GPC and Z protein) have been developed, but the high background levels in African sera of antibodies against components of bacterial or insect all expression systems has complicated their use in endemic regions. Development work continues.

Specific Lassa IgM antibody may persist for months to years, and IgG for decades.

#### 4.3.6. Whole genome sequencing

Whole genome sequencing using next generation sequencing technologies (eg. Illumina and Oxford Nanopore) can be utilised to confirm the molecular detection of LASV and support contact tracing efforts through detailed phylogenetics and genomic epidemiological analysis. Unlike traditional molecular diagnostic assays, whole genome sequencing using shotgun or pan-viral hybridization/capture metagenomic approaches is highly tolerable to viral genome diversity and evolution, providing a useful tool for monitoring molecular diagnostic assay escape during an outbreak (27-29).

### 4.4. Lujo virus

#### 4.4.1 Samples

Serum or plasma is the most commonly used diagnostic sample for nucleic acid testing and virus isolation. Virus may also be recovered from post-mortem liver biopsies.

### 4.4.2 Nucleic acid testing

In-house RT-PCR and real-time RT-PCR methods are available for detecting the Lujo virus. The former enables sequence confirmation and viral characterisation

### 4.4.3 Virus isolation by cell culture

Lujo virus can be cultured in Vero-E6 cells, although cytopathic effect is not obvious. PC4 containment is required for virus amplification in cell culture which may be undertaken for confirmatory testing, reagent production, test validation or research.

### 4.4.4 Antibody detection

Serology assays are currently unavailable for antibody detection of Lujo virus.

### 4.4.5 Sequencing

Sanger and Next Generation Sequencing methods can be employed to confirm Lujo virus.

## 4.5. Rift Valley fever virus

### 4.5.1. Samples

Samples collected during the febrile stage of illness are preferrable for viral identification. Blood collected in anticoagulant (EDTA) is the preferred specimen for RVFV nucleic acid testing, but serum or plasma can also be used. Virus may also be detected or isolated in other body fluids and tissue. Serology testing is performed on serum or plasma and antibodies are detectable from 5-6 days after the onset of disease (30).

### 4.5.2. Nucleic acid testing

RT-PCR is the most sensitive and specific method for detecting RVFV during the acute phase of infection, when viral load is high. Viral RNA has been detected in a range of clinical samples including blood, serum, plasma, urine, and tissues

### 4.5.3. Virus isolation by cell culture

Virus isolation, at PC4 level, from whole blood, serum, plasma or other clinical material remains a reference method for confirmatory testing, reagent production, test validation or research. RVFV isolation can be performed in different cell lines, including Vero E6, baby hamster kidney (BHK) and AP61 mosquito cells. Cytopathic effect is evident in Vero-E6 cells. Confirmation of virus isolation should be performed using IFA or by real-time RT-PCR.

#### 4.5.4. Antibody detection

The viremic period of RVFV is typically very short (4-6 days), thus presenting a very limited window of detection with the antigen detection assays or molecular tests like RT-PCR. Combining nucleic acid testing (NAT) with serological detection (IgM capture ELISA/IFA) are generally preferred for the diagnosis of RVFV, particularly for individuals who are no longer in the viremic phase of infection (30).

Commonly used serological assays include ELISA, IFA and VNA. Commercially available ELISA kits are often employed for these serological tests, providing a reliable method for detecting RVFV antibodies (31). However, there is currently no Australian Register of Therapeutic Goods (ARTG)-registered serological assay for RVFV. Most ELISA-based assays for RVFV are designed to detect antibodies targeting the virus's nucleocapsid protein (N protein) (32). This protein is highly conserved and immunogenic, making it an ideal target for serological assays. It has been reported that RVFV-specific IgM antibodies can remain in the bloodstream for up to 6 weeks (33).

#### 4.5.5. Sequencing

Confirmation of RVFV can be done by targeted sequencing of the M section of the glycoprotein gene. Targeted sequences of the non-structural (Ns) and glycoprotein (Gn) gene are used for phylogenetic analysis (34). Although amplicon-based WGS schemes have been described, few have been validated fully on clinical specimens (35, 36). Hybridisation/capture and/or shotgun sequencing using Illumina and Oxford Nanopore technologies can be employed to provide confirmation of RVFV.

### 4.6. New World (NW) arenaviruses (Junin virus, Machupo virus, Guanaritovirus, Sabia virus and Chapare virus).

#### 4.6.1 Samples

Serum, plasma, urine, throat wash samples and human tissue can be used for laboratory testing of New World arenavirus infections.

#### 4.6.2 Nucleic acid testing

Detection of NW arenavirus RNA can be by conventional RT-PCR and/or virus-specific real-time RT-PCR.

#### 4.6.3 Virus isolation by cell culture

Virus isolation can be achieved through cell culture (e.g.Vero cells)

### 4.6.4 Antibody detection

Serology assays are currently unavailable for antibody detection of New World Arena viruses.

### 4.6.5 Sequencing

Sanger sequencing of PCR products from conventional RT-PCR can be used to confirm the virus type. Custom-designed and commercial kits employing next-generation sequencing (NGS) technologies are also available for the identification and characterisation of NW arenaviruses.

# 5 Quality assurance

The RCPA Quality Assurance Program has in recent years developed a QAP module (Viral Specimen Module) covering proficiency testing for VHF viruses in which Australian testing laboratories may enrol. The modules comprise of 2 surveys per year with up to 4 samples per survey. Simulated specimens consisting of total viral RNA and RNA transcript mixtures for target genes of EBOV (GP, L(RdRp), NP, VP40), and RNA transcript mixtures for target genes of MARV (GP, L(RdRP), NP, VP35) have been circulated and tested by participants. Simulated Lassa fever specimens comprised total viral RNA from inactivated Lassa virus culture supernatant. Simulated Rift Valley fever specimens comprised total viral RNA from inactivated RVFV culture supernatant.

# 6 Laboratory nomenclature for national data dictionary

| SNOMED CT code | Concept name | Description |
|---|---|---|
| 240523007 | Viral Haemorrhagic fever | Disorder |
| 37109004 | Viral haemorrhagic fever, Ebola | Disorder |
| 77503002 | Viral haemorrhagic fever, Marburg | Disorder |
| 772158005 | Viral haemorrhagic fever suspected | Situation |
| 402917003 | Viral haemorrhagic fever, Rift Valley | Disorder |
| 43489008 | Viral haemorrhagic fever, Crimean-Congo | Disorder |

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# 8 Glossary

**Ag/Ab** – Antigen/Antibody

**CDNA** – Communicable Diseases Network Australia

**CSF** – Cerebrospinal fluid

**Ct** – Cycle threshold

**DNA** – Deoxyribonucleic acid

**EDTA** – Ethylenediaminetetraacetic acid

**EIA** – Enzyme immunoassay

**ELISA** – Enzyme linked immunosorbent assay

**IFA** – Immunofluorescent antibody

**IgA** – Immunoglobulin A

**IgG** – Immunoglobulin G

**IgM** – Immunoglobulin M

**IVD (device)** – *In vitro* diagnostic medical device

***In vitro*** – performed in a test tube, culture dish, or elsewhere outside a living organism

***In vivo*** – performed or taking place in a living organism

**ITS** – Inter-genic spacer region

**NAT** – Nucleic acid amplification test/ing

**NATA** - National Association of Testing Authorities, Australia

**NGS** – Next generation sequencing

**NPAAC** – National Pathology Accreditation Advisory Council

**NRL** – National Serology Reference Laboratory

**PCR** – Polymerase chain reaction

**PC2 laboratory** – Physical containment level 2 laboratory

**PC3 laboratory** – Physical containment level 3 laboratory

**PC4 laboratory** – Physical containment level 4 laboratory

**QAP** – Quality assurance program

**QC** – Quality control

**RT** – Reverse transcriptase

**RT-PCR** - Reverse transcription polymerase chain reaction

**RT-qPCR** – (real-time quantitative reverse transcription polymerase chain reaction

**SSBA** – Security sensitive biological agent

**Strain** – Variant that possesses unique and stable phenotypic characteristics

**Test specificity** – Ability of a test to correctly identify people without the disease

**TGA** – Therapeutic Goods Administration

**VTM** – Viral transport media

**WGS** – Whole genome sequencing

**WHO** – World Health Organization