

Viral haemorrhagic fever

Laboratory case definition

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

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1 PHLN Summary Laboratory Definition

1.1 Condition:

Acute Viral Haemorrhagic Fever (VHF), due to the quarantenable VHF viruses *Ebola*, *Marburg*, *Lassa*, *Crimean-Congo*.

1.1.1 Definitive Criteria

- Detection of VHF virus RNA and/or antigen in clinical material, with at least two separate RNA or antigen targets positive and confirmation by nucleic acid sequencing of one target.
- Isolation of VHF virus from clinical material, confirmed by RT-PCR or antigen detection.
- Detection of VHF virus antibody seroconversion, or a fourfold or other significant rise in a quantitative VHF virus serology test.

1.1.2 Suggestive Criteria

- Detection of VHF virus single RNA/antigen target.
- Detection of VHF virus IgM, with or without detection of IgG.

1.1.3 Comments

Requests for testing should come endorsed by the Chief Health Officer of the state or territory once satisfied that grounds for testing exist i.e. following risk assessment (ref to CDNA SoNG). A single suspected VHF case meeting these criteria is an emergency requiring immediate notification to Commonwealth and jurisdictional public health authorities.

2 Introduction

2.1 Viral Haemorrhagic Fevers

Viral haemorrhagic fever is a clinical syndrome classically associated with fever and an abnormal tendency to bleed. Viral causation of VHF is diverse involving members of four viral families: *filoviruses*, *bunyaviruses*, *arenaviruses* and *flaviviruses*. The subgroup of VHF for which person to person transmission potential has been demonstrated are regarded as especially serious, and are quarantineable diseases in Australia. The quarantineable VHF are the *filoviruses* *Ebola* and *Marburg*, the *arenavirus* *Lassa fever*, and the *bunyavirus* *Crimean-Congo virus*. Infections with *Lassa virus* are believed to be relatively frequent, occurring year round in West Africa. In contrast human infections with *Crimean Congo virus* are quite infrequent. The largest VHF outbreak was caused by *Ebola virus* in 2014/15.

VHF viruses are enveloped RNA viruses. They are maintained in nature in animal hosts, some yet to be definitely identified, spreading zoonotically to humans. The viruses are geographically restricted by the distribution of their natural host. 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 *Ebola virus*, and Crimean-Congo viruses and uncommon for Lassa. Infections may be severe and life threatening.

2.2 *Ebola Virus*

Ebola emerged in 1976 almost simultaneously in the Democratic Republic of Congo (then Zaire), and Sudan. *Ebola* and the closely related *Marburg Virus* are genera within the family *filoviridae*. The genus *Ebolavirus* has 5 species varying by 30-40% at the nucleotide level. *Ebola* Zaire (ZEBOV), *Ebola* Sudan (SEBOV), *Tai Forest* (ICEBOV, formerly Ivory Coast) and *Bundibugyo* (BEBOV) and *Reston* (REBOV). *Reston* has caused VHF in non-human primates but not in humans. *Filoviruses* are enveloped filamentous RNA viruses with virions of variable length typically 80nm x 100nm in 'shepherd's crook' or 'eye-bolt' sharps. The RNA genome is negative sense, 19kb in length with a linear arrangement of 7 genes coding for at least 9 proteins including a RDRP replication complex (L,VP35) and an envelope glycoprotein (GP).

The natural reservoir of *Ebola* remains unknown, but serologic evidence suggests three species of fruit bat in Africa (*Hypsignathus monstrosus*, *Epomops franqueti* and *Myonycteris torquata*) and in *R. amplexicaudatus*, a common species of fruit bat in the Philippines. *Ebola virus* 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 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.

Ebola cases 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 rates have 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 scale, in its West African location, and in its extent of international spread. This outbreak involved in excess of 28,000 known cases and more than 11,000 deaths. It began in Guinea in Gueckedou province near the borders with Liberia and Sierra Leone in December 2013. The outbreak spread in 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 health care 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 3000 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 (20 cases), Mali in October 2014 (8 cases) and Senegal in August 2014 (1 case). Infected nationals, mainly health care workers, were repatriated to Spain (2), USA (6), Germany (2), France (2), Norway (1), UK (1), Switzerland (2) and Italy (1). There was also secondary spread to healthcare workers from repatriated cases in the USA (2) and Spain (1). A concerted and prolonged international effort was required to contain and end the outbreak.

There have been 5 documented laboratory accidents involving *Ebola virus*. All were in a research setting. Three involved needlesticks to researchers while working with animals (2004, USA, no infection; 2005 Russia, fatal infection; 2009 Germany, remained well treated with VSV/ZEBOV experimental vaccine). A fourth (1976, UK, survived treated with interferon and convalescent plasma), suffered a needlestick 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.

Ebola 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, *Ebola Reston* infection was confirmed in pigs in the Philippines for the first time.

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

2.3 Marburg Virus

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 in Germany, and several weeks later 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%). One of the 32 cases was diagnosed retrospectively by serology. 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. The same Ugandan primate exporter was implicated

as the source of all three outbreaks. There were subsequently conflicting reports regarding the origin of the involved primates. 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. The wife of the Belgrade index case was a physician and had drawn blood at home for testing and so this case has been categorized as nosocomial transmission. The case of suspected sexual transmission occurred more than 12 weeks after the recovery of a male Behringwerke employee involved in the primary outbreak. *Filovirus* antigen was detected in his serum by immunofluorescence. A novel virus, named *Marburg virus* (MARV) was isolated from blood and tissues of infected patients. *Marburg* is a *filovirus* with basic virological properties as described above for *Ebola virus*.

Marburg virus is indigenous to Africa and while the geographic areas in which it is endemic are unknown, they appear to include at least parts of Uganda, Western Kenya, Angola and perhaps Zimbabwe. Recent studies implicate the African fruit bat *Rousettus aegyptiacus* as the reservoir host of the *Marburg virus* but further study is required to determine if there are other host species. The fruit bat is widely distributed across Africa, extending the area at risk for outbreaks of Marburg haemorrhagic fever beyond that previously suspected.

After the initial 1967 outbreak the next known cases of *Marburg virus* infection did not occur until 1975 in Johannesburg. The patient, a 20 year old Australian student was fatally infected having most likely been exposed while travelling in Zimbabwe. His travelling companion and a nurse were subsequently infected but survived. In 1980, a French engineer who travelled in western Kenya was infected and died. A physician who cared for the case in Nairobi hospital was infected but recovered. *Marburg virus* was grown from clinical specimens, and detected in semen two months after recovery. In 1987 a Danish boy who visited Kitum caves near to where the engineer was infected was also infected and died. There were no secondary cases. In 1982, a case was identified in an 18 year old from the same rural part of Zimbabwe in which the case from 1975 had stayed. This patient recovered and there were no secondary cases.

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.

Outbreaks have subsequently been reported in the Democratic Republic of Congo (1998-2000, 154 cases, 128 fatal), Angola (2004-5 252 cases, 227 fatal) and Uganda (200, 2 cases, 1 fatal), predominantly among mine workers. In 2008, two cases (one death) were reported in tourists, one Dutch and the other American, returning from Uganda. Both travelers had visited Kitum cave inhabited by fruit bats in a national park.

While the *Marburg* case fatality rate was initially thought to be significantly lower than that of Ebola, analysis of recent outbreaks in the Democratic Republic of Congo has shown that this is also greater than 70%. A case fatality rate of 90% was documented in an outbreak in Angola in 2004-2005. Recovery from *Marburg* can be slow and known sequelae include orchitis, recurrent hepatitis, transverse myelitis and uveitis.

2.4 Crimean-Congo Virus

What is now known as Crimean-Congo haemorrhagic fever (CCHF) was first described in the Crimea in 1944 during a large outbreak of severe haemorrhagic fever in agricultural workers (200 cases, with a case fatality rate approximately 10%). In 1969 after viral and serological studies it was recognised that the virus identified as the cause of Crimean haemorrhagic fever was the same as that cultured from a case of febrile illness identified in 1956 in the Congo. The viruses were subsequently renamed *Crimean-Congo virus* (CCV).

Crimean-Congo virus is a member of the genus *Nairovirus*, family *Bunyaviridae*. *Bunyaviruses* are enveloped viruses approximately 100nm in diameter. They are generally spherical but may be oval or elongated. The core contains a segmented negative sense single stranded RNA genome with short (S), medium (M) and long (L) genome segments of 1.7, 4.9 and 12.2 kb, total 18.8kb in length. These code for the nucleocapsid (NP), two envelope proteins (Gn and Gv) and the viral transcriptase proteins. The viral genome is highly variable between CCHF isolates, with 20, 31 and 22% nucleotide differences reported for the S, M and L genes respectively. In contrast the deduced amino acid difference in the NP is only 8%, and isolates are regarded as representing a single serotype.

Crimean-Congo virus, 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. The majority of cases have occurred in the livestock industry including agricultural workers, slaughterhouse workers and vets. However, CCV has repeatedly caused nosocomial outbreaks with high mortality rates among healthcare workers, and laboratory workers. Percutaneous exposure represents the highest transmission risk, estimated at 33% in one study. Tertiary cases amongst family members of healthcare workers are also well described. Between 1950 to 1974 in Bulgaria, 42 health care workers were infected with CCV with a fatality rate of 40%. The case fatality rate among 14 healthcare workers in Turkey from 2003 and 2009 was 28%.

CCHF 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.

In Europe, CCHF 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 CCHF 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. 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 fever was first recognised in 1969 after transmission of a severe systemic disease from a missionary nurse infected in Lassa, Nigeria, first to the nurse caring for her and subsequently to a third nurse who was the only survivor after evacuation to the USA. A virologist from the Yale Arbovirus Research Unit was also infected in the course of the episode. A new *arenavirus*, named for the source location was isolated from these patients.

Lassa virus is an *arenavirus* (Family *Arenaviridae*, genus *Arenavirus*); classified on antigenic and molecular properties within the Old World group along with *Lymphocytic Chorio Meningitis virus* (LCMV) and others. *Arenaviruses* are enveloped and pleomorphic including round and oval particles ranging in size from 40 to greater than 200nm. 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 natural hosts of *arenaviruses* are rodents, and there is high species specificity between each virus and its rodent host. In the case of *Lassa virus* the natural hosts are *Mastomys* species (multimammate mice). The rodent hosts of *arenaviruses* are chronically infected and the viruses are shed into the environment in the urine or droppings. Vertical infection maintains continuity of infection among 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 excrement, as well as human to human spread which is believed to be common in the village setting. *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 extensive human infection, including occasional nosocomial outbreaks have been reported from Nigeria, Liberia, Sierra Leone, and Guinea. 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 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.

3 Tests

National guidelines for VHF specific viral diagnosis from the USA, Canada and the UK and the EU still emphasize centralisation of diagnostic capacity in national reference facilities equipped with Physical Containment Level 4 (BSL-4) laboratories. Guidelines in the UK and EU provide for screening of some low-risk patients in hospital laboratories. In contrast large numbers of *Ebola virus* diagnostic tests have been done in the field in much simpler facilities during the West African Ebola outbreak of 2014/15.

In Australia the safety and quality of VHF diagnostic testing will be well served by limiting testing to PHLN member laboratories with high quality facilities, equipment and staff, together with the capacity to authoritatively assess and effectively manage biosafety risk.

The National High Security Quarantine Laboratory (NHSQL) at VIDRL is Australia's designated national reference laboratory for quarantineable VHF. It provides diagnostic capability, and confirmatory testing on an urgent on-call basis 24/7.

3.1 Access to Diagnostic Testing

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

NHSQL at VIDRL is available to provide primary diagnosis 24/7, which it does for the majority of jurisdictions. Where testing is done at a jurisdictional level NHSQL is available to parallel test, or confirmatory test results 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 on mobile 0438 599 437. If attempts to reach the VIDRL on call medical microbiologist via these numbers fail the designated back up is the VIDRL on call laboratory manager 0438 599 439, or failing this the Royal Melbourne Hospital switchboard (03) 9342 7000 who will attempt to reach VIDRL staff on the caller's behalf.

PCR is the primary diagnostic modality employed for detection of Ebola 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 a sample of venous blood, and an oral swab. If post mortem specimens are available, serum, liver, spleen and kidney tissues are desirable.

Appropriate equipment includes:

- Personal protective equipment
- 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 clean the outside 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. Ten millilitres of clotted blood or blood anti-coagulated with EDTA (NOT heparin) should be placed in a sealed plastic container. Needles should not be recapped, bent, broken, removed from disposable syringes or otherwise handled. 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 1ml of sterile, viral transport medium (Minimum Essential Medium plus 2% foetal calf serum, penicillin 100 units/ml, streptomycin, 100 ug/ml neomycin 40ug/ml and amphotericin B 20 ug/ml; available from VIDRL on request) or equivalent transport medium. A dry swab is preferable if no VTM is available.

3.1.2 Transport of Specimens to the Laboratory

The outside of each specimen container should be swabbed 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, airtight and watertight bags, which have been similarly labelled. Bags containing specimens should be sponged with disinfectant before being removed from the patient's room.

Samples should be identified as: "Infectious Substances Affecting Humans (Ebola sample)" and packaged and handled as required by the International Air Transport Association (IATA) packing instruction 602.

The specimens should be packaged as follows:

- Place the specimens for transport in tightly sealed, watertight container, such as a screw-cap plastic tube or vial. Ensure plastic containers are resistant to temperatures as low as -800C.
- Wrap the primary container in sufficient absorbent material (e.g. tissue) to absorb the entire contents in case the container leaks or breaks.
- Place the wrapped, sealed primary container into a durable, watertight, screw-cap plastic container.
- Several primary containers may be placed in one secondary container to a maximum of 50mL of specimen material.
- On the outside of the secondary container, attach the specimen labels and other relevant information.

Place the second container in a secure box or mailing tube addressed to:

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

Use a competent door-to-door courier. 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. For virus amplification in cell culture PC4 containment is mandatory. 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 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 high quality 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 [Commonwealth Department of Health's 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* and *Marburg*

Although there is more published experience regarding *Ebola virus* than for *Marburg virus*, the similarity of the two viruses and their respective clinical syndromes justify extrapolating from *Ebola* to *Marburg* and treating the two together as a single entity.

4.1.1 Samples

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

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. This and the desirability of sampling from a second site to increase robustness of diagnosis has led to exploration of use of oral fluid. Initial data suggests that while the detection rate in oral fluid is not as high as from serum or plasma, that it is a worthwhile supplementary specimen.

4.1.2 Nucleic Acid Testing

As for all of the quarantenable VHF nucleic acid testing, performed primarily on serum or plasma, has become the preferred diagnostic method for *Ebola* and *Marburg viruses*.

A variety of conventional, and more recently real-time RT-PCR assays have been developed targeting the Nucleoprotein (NP), Glycoprotein (GP), or occasionally L or RdRP genes of the two *filoviruses*. They have been shown to be sensitive and specific, including under field conditions in the laboratory diagnosis of *Ebola virus* in particular where a relatively higher frequency of recent outbreaks has produced more published experience.

Viraemia can be reliably detected from the time of presentation for at least 14 days; occasionally for as long as 21 days. Because of a small number of false negatives early in the clinical course negative results obtained in the first 48 hours of illness should prompt testing of a second specimen obtained after this time when a high degree of clinical suspicion exists.

Quantitative RT-PCR analysis has demonstrated viral loads ranging from 10⁴/ml to 10¹⁰/ml. Viral load appears to correlate with clinical outcome, with fatal cases shown to have viral loads throughout disease that averaged 2 log₁₀ higher than in those who survived. Average peak titre in fatal cases has been described as 3.4 x 10⁹/ml compared to 4.3 x 10⁷/ml in survivors. Virus load measured by RT-PCR has been demonstrated to be 3-4 log₁₀ higher than the corresponding measurement in cell culture by plaque assay.

Inhibition controls are important within VHF RT-PCR assays to guard against false negatives. It is thought that tissue destruction releases assay-inhibitory substances into blood. In one example despite a virus load of 6.9 x 10⁸ a moribund Ebola patient recorded a negative RT-PCR result which became positive when a 100-fold dilution was subsequently tested.

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 productive 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 and MA-104 cells, although *filoviruses* will grow in a range of cells. Cell culture is relatively sensitive, although cytopathic effect can be variable. Viral growth can be monitored by immunofluorescence or RT-PCR.

The *filoviruses Ebola* and *Marburg* are Tier 1 Security Sensitive Biological Agents (SSBA) in Australia. They may only be stored or manipulated in an appropriately accredited facility by authorised staff.

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. While the analytical sensitivity of antigen detection may be expected to be lower than RT-PCR, its clinical sensitivity has still been relatively good, likely due to the relatively high titre and sustained

viraemia. Antigenaemia has been shown to clear with the appearance of IgM on average 1-2 days before RT-PCR became negative.

As is the case for other VHF, antigen detection is being supplanted by nucleic acid testing both in the laboratory and in the field. Many laboratories still maintain antigen detection capability as a second-line test however. In addition a variety of relatively inexpensive, simple rapid antigen detection tests have been developed in response to the West African *Ebola virus* outbreak; a number in time to undergo field evaluation during the outbreak.

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 cell culture 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 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 all been produced as a basis for serologic assays. Assays based on NP of both Ebola and to a lesser extent *Marburg viruses* have been evaluated using human and non-human primate sera. Assays based on GP and VP35 antigens have also been developed. Commercial EIA reagents now exist, but may not be available in Australia.

4.1.6 Electron Microscopy

Because *Ebola virus* is commonly present in sufficiently high titres in serum and plasma it may be possible to visualise directly in a patient sample using electron microscopy. In the past this has provided some rapid diagnostic capability in appropriately equipped laboratories. Although this is rapid it is unlikely to have continuing diagnostic utility as a primary diagnostic approach given the relative speed and simplicity of nucleic acid testing.

4.2 *Crimean Congo 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 quarantenable VHF nucleic acid testing has become the mainstay of diagnosis for CCHF. Reverse transcription polymerase chain reaction (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, especially in patients originating from the Balkans and Turkey. 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 10⁸ copies/ml in plasma can be considered predictive of fatal outcome.

4.2.3 Viral Isolation

Virus isolation remains a reference method having been surpassed in sensitivity, speed and convenience for diagnostic purposes by nucleic acid testing. PC4 containment is required for virus amplification in culture for confirmatory testing, reagent production, test validation or research. CCV replicates in a variety of cell lines including Vero, chicken embryo lines, BHK-21 and primary calf kidney. The virus is partly cytopathic. After 1-6 days of culture confirmation of growth by IFA or RT-PCR is usually required. Isolation generally only detects the relatively high levels of viraemia 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, 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%).

4.3 *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 strains. In a diagnostic context increased turnaround times, and risk of contamination are inherent disadvantages compared to the rapidity and closed tube format of real time PCR. Real time PCR using Sybr 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 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 as long as day 21. Viraemias of between 10⁶ and 10⁹ RNA copies/ml have been described. PCR inhibition due to substances present in VHF patient blood has been described for Lassa fever along with Ebola virus disease and yellow fever. 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, especially with passaging. PC4 containment is required for virus amplification in 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, but in a proportion of patients virus has been isolated as late as day 21.

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 week 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 immunofluorescence using virus-infected cells as antigen has been the most common serological test method. A fourfold 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 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.

5 Quality Assurance

The RCPA Quality Assurance Program has in recent years developed a QAP module covering proficiency testing for African VHF in which Australian testing laboratories may enrol. Simulated specimens consisting of RNA transcripts for target genes of Ebola (GP1, GP2, L, NP, VP40), and *Marburg* (GP, L, NP, VP35) have been circulated and tested by participants. Simulated Lassa specimens comprised gamma-irradiated cell culture grown *Lassa virus* (Pineo Nigeria strain).

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