Diagnostic Testing for Crimean-Congo Hemorrhagic Fever

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ABSTRACT Crimean-Congo hemorrhagic fever is the most geographically widespread tick-borne virus, with infection resulting in mortality in up to 30% of cases. Clinical diagnosis alone is difficult due to the nonspecific nature of symptoms; therefore, laboratory diagnostics should be utilized for patients with residence in or travel to regions of endemicity in whom the disease is suspected. This minireview provides an overview of laboratory tests available for Crimean-Congo hemorrhagic fever (CCHF) and their utility in diagnosis with a focus on diagnosing CCHF in humans.

KEYWORDS Crimean-Congo hemorrhagic fever virus

Crimean-Congo hemorrhagic fever virus (CCHFV) is an enveloped RNA virus in the *Orthonairovirus* family of the order *Bunyavirales* (1). The virus is primarily spread through the bite of infected ticks of the *Hyalomma* genus, although it may also be transmitted through direct contact with body fluids from infected animals and humans or spread via improperly sterilized medical equipment (2–4). A large variety of animals, including cattle, cynomolgus monkeys, donkeys, goats, ground squirrels, hares, horses, hedgehogs, ostriches, small rodents, and sheep, develop viremia after exposure to CCHFV (5, 6). However, the majority of animals do not develop symptoms of illness following CCHFV infection, with the exception of horses, sheep, cynomolgus monkeys, and neonatal or immunocompromised mouse models (5, 6). Viremia in animals is short lived, making viral detection difficult, as most animals are asymptomatic during this period (5). In contrast, the presence of CCHFV may be directly detected in *Hyalomma* ticks using viral antigen testing, nucleic acid amplification testing, or nucleic acid amplification testing combined with proteomics (7–11). Serological studies are the main diagnostic test used for CCHFV infection surveillance in animals. A meta-analysis examining the seroprevalence of anti-CCHF antibodies in animals, primarily livestock, worldwide demonstrated a mean seroprevalence of 24.6%, indicating animal exposure to CCHFV is relatively common, with an uptrend in seroprevalence in more recent years (12).

CCHFV is the most geographically widespread tick-borne virus, with regions of endemicity including parts of Africa, Asia, Eastern Europe, the Middle East, Russia, and Spain (3, 13–15). An estimated 10,000 to 15,000 cases of CCHF occur annually, mainly in endemic countries, with only rare cases reported among travelers (21 travel-related cases reported as of 2016) (13, 16). A meta-analysis of human serological studies revealed a mean seroprevalence of anti-CCHF antibodies of 4.7%, with an uptrend in seroprevalence in more recent years (12). Additionally, the seroprevalence of anti-CCHF antibodies is 7.5-fold higher among persons with high-risk exposures, including tick bites, contact with a human CCHF case, or occupations involving frequent animal exposure (12). Increasing trends in CCHFV seroprevalence in both humans and animals, combined with evidence that people with frequent animal contact have more exposure to CCHFV, raise the concern that CCHFV could emerge as a zoonotic pathogen (12).

The RNA of CCHFV is divided into three segments; the S segment encodes the nucleocapsid protein, the M segment encodes glycoproteins, and the L segment

encodes an RNA-dependent RNA polymerase. CCHFV demonstrates a wide degree of genetic diversity, with strains divided into seven clades, primarily based on the genetic relatedness of the S segment (17–19). These clades cluster geographically, with three clades primarily occurring in Africa, two in Asia, and two in Europe (17–19). The degree of nucleotide variation between strains can reach up to 20% for the S segment, 31% for the M segment, and 23% for the L segment (17).

CLINICAL PRESENTATION AND TREATMENT

The majority of infections with CCHFV are subclinical (13). Patients who develop symptomatic CCHF progress through the following four stages: an incubation stage, a prehemorrhagic stage, a hemorrhagic stage, and a convalescent stage. The asymptomatic incubation period lasts between 1 and 13 days (4). Symptoms begin abruptly in the prehemorrhagic stage and are not specific to CCHFV and, instead, commonly mimic other bacterial infections, viral infections, and parasitic infections, such as malaria. Symptoms may include fevers, chills, myalgias, dizziness, headache, mood swings, eye soreness, photophobia, sore throat, neck pain and stiffness, lymphadenopathy, and gastrointestinal symptoms, including nausea, vomiting, abdominal pain, and diarrhea (4, 20, 21). The diagnosis of CCHF can easily be missed in the prehemorrhagic stage, leading to delays in treatment initiation. In one study from Turkey, 68% of CCHF patients were initially misdiagnosed, leading to delayed hospital admission and higher mortality rates than those correctly diagnosed at the time of initial presentation to medical care (22). The hemorrhagic stage commonly develops 3 to 5 days after the onset of symptoms with bleeding manifesting as petechiae, ecchymosis, and mucosal bleeding, including from the gastrointestinal or genitourinary tracts, and/or internal bleeding (4, 20, 21). Mortality occurs in approximately 30% of cases, often in the second week of illness (4). Convalescence usually begins among survivors after 9 to 10 days (21). Supportive treatment is the mainstay of therapy. Ribavirin, an antiviral medication, has been administered to many patients with CCHF, although its effectiveness remains unclear (23). Other therapeutic interventions utilized in case reports and series include high-dose steroid administration, transfusion of plasma from convalescent patients, intravenous immunoglobulin administration, and plasma exchange, although insufficient evidence is available to assess the effectiveness of these interventions (24).

OVERVIEW OF DIAGNOSTIC TESTING

A variety of laboratory assays may be used to diagnosis CCHF, and the biosafety level (BSL) requirements for performing diagnostic testing vary by assay type and by country. In some countries, including France, Germany, Italy, Sweden, Switzerland, and the United Kingdom, BSL4 precautions are recommended for the performance of diagnostic assays for CCHF, while other countries recommend BSL3 or BSL2 precautions (25, 26). Biosafety requirements may be higher for researchers using live CCHFV than laboratories performing only diagnostic testing. Inactivation of CCHFV in samples may be performed to minimize risk if high containment settings are not used for diagnostic testing. Inactivation methods include heating to 56°C for at least 30 minutes, gamma irradiation, UV light, acidifying the pH to less than 6, and the addition of fixatives or disinfectants, including 1% hypochlorite, 2% glutaraldehyde, formalin, paraformaldehyde, 1% sodium hypochlorite, hydrogen peroxide, and peracetic acid (27–29). Other chemical inactivation methods include the addition of 100% alcohol and AVL buffer or buffer RLT (Qiagen, Valencia, CA), 0.5% Tween 20 (Thermo Fisher, Waltham, MA), SDS, TRIzol LS (Invitrogen Corp., Carlsbad, CA), and potentially Triton X-100 (Sigma-Aldrich, St. Louis, MO) (28, 30). Care should be taken when choosing an inactivation method to avoid interference with the diagnostic assay being used.

The diagnosis of human CCHF may be confirmed by directly detecting the presence of CCHFV or by measurement of serological responses consistent with acute infection (anti-CCHFV IgM antibodies or a 4-fold increase in anti-CCHFV IgG titers between serial blood samples). Factors to be considered when deciding which type of assay to deploy include the duration of time elapsed since symptom onset and the validity of the test
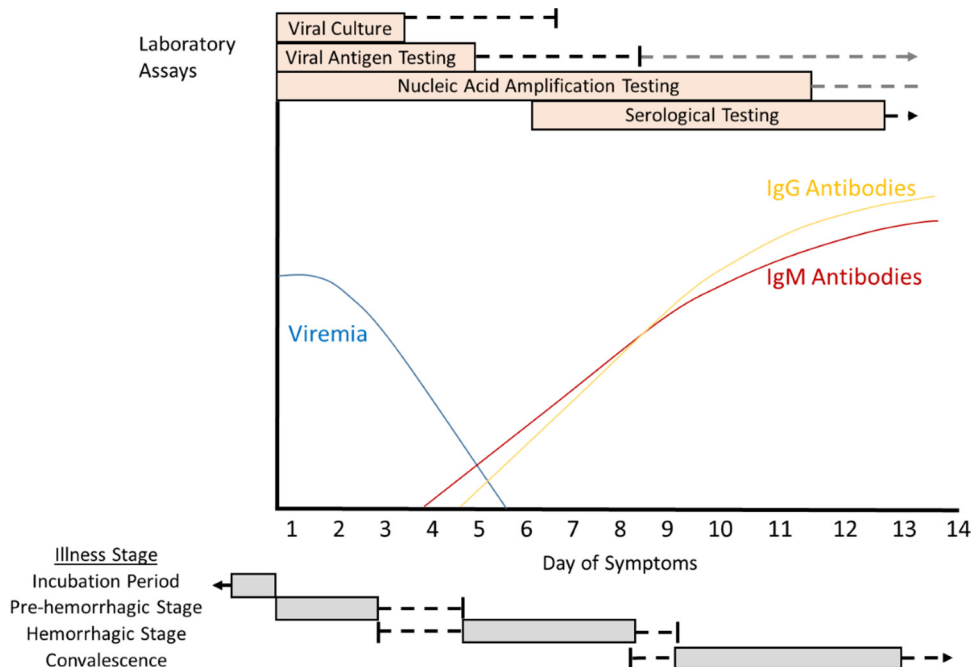
FIG 1 Cartoon overview of diagnostic testing for acute illness due to Crimean-Congo hemorrhagic fever virus in a non-fatal human infection. Patients with fatal CCHFV infections may have prolonged viremia and may not develop antibodies to CCHFV. The period of greatest assay utility is shown in the orange box. Black lines indicate the outer limits of utility in patients who recover, gray lines indicate periods of uncertain positivity or documented positivity among fatal cases, and black arrows indicate persistent assay positivity. The typical periods of each stage are outlined in gray boxes, with ranges outlined by black lines.

for the CCHFV strains found in the suspected region of exposure. A cartoon overview visually depicting the illness stages of CCHF in a nonfatal human case, viral and humoral immune dynamics, and periods of diagnostic assay utility is shown in Fig. 1.

Assays that directly assess for CCHFV infection, including viral culture, nucleic acid amplification tests, and viral antigen detection assays, are most useful during the first week after symptom onset. Viremia is most common in the first few days after symptom onset but rapidly wanes over the first week of illness (20, 21, 31–34). In one case series, CCHFV viremia was detectable for a mean of 4 days (range, 1 to 6 days) after admission among hospitalized patients (32). Occasionally, viremia may be detected after the first week of illness; this is more common among severe cases resulting in a fatal outcome (31–34). Viremia resolves around the time anti-CCHFV antibodies develop (31, 34, 35).

Serological testing for the diagnosis of CCHF is most likely to be useful after the first week of illness. IgM antibodies become detectable 7 to 9 days after symptom onset, although they may rarely be detectable as early as day 4 of illness (20, 31, 34–37). Administration of intravenous immunoglobulin does not affect the timing of IgM antibody development (36). IgM antibodies peak 2 to 3 weeks after symptom onset and decline to low levels by 4 months (35, 36). IgG antibodies become detectable simultaneously or within 1 to 2 days after IgM becomes detectable (31, 35). IgG antibody levels peak 2 to 3 weeks after symptom onset in some patients and after 2 to 5 months in others, with IgG antibodies remaining detectable for at least 3 years after infection (35, 36). Some CCHF patients with fatal outcomes do not develop detectable antibodies to CCHFV (20, 34). In patients with negative CCHFV antibody testing in the second week of illness, especially in the setting of persistent severe symptoms, direct viral testing may be warranted in addition to serological testing to avoid misdiagnosis.

Multiple biomarkers are being investigated for the diagnosis of CCHFV infection; however, they have not yet been validated for diagnostic use.

ASSAYS FOR VIRAL DETECTION

Viral culture. Viral culture may be performed using cell lines from chickens, hamsters, and monkeys (specifically BHK-21, CER, LLC-MK2, and Vero cell lines) combined with either fluorescence focus or plaque-based assays or may be performed via intracerebral inoculation of mice (28, 33, 38). Animal inoculation is 10- to 100-fold more sensitive than cell culture; however, this procedure takes 5 to 10 days to achieve a result compared with 1 to 6 days with cell culture in CER cell lines (33). However, some CCHFV strains produce little cytopathic effect and may require additional techniques, such as nucleic acid amplification tests or immunofluorescence assays, to confirm the presence of virus (33). A pseudoplaque assay utilizing an enzyme-catalyzed color change to detect cellular infection has a higher sensitivity for detection of CCHFV than immuno-fluorescence (39).

Viral culture is most effective and likely to yield a faster time to positivity if performed early after the onset of symptoms when high levels of viremia are most common (33). One advantage of using viral culture for CCHF diagnosis is its capability to detect a wide diversity of CCHFV strains. Disadvantages include taking several days to yield a result and biosafety limitations requiring viral culture to be performed using maximal biosafety precautions, often in BSL3 or BSL4 laboratory facilities, as samples cannot be inactivated prior to culture (40). As high containment laboratory facilities are not available in many regions of endemicity, access to viral culture as a diagnostic method is often unavailable.

Nucleic acid amplification tests. Many nucleic acid amplification tests have been published for CCHFV diagnosis (Table 1), although few tests are commercially available (7, 9, 11, 37, 41–58). Reverse-transcription PCR (RT-PCR) is commonly used for the diagnosis of CCHVF in the first 10 to 12 days after symptom onset and is generally more widely available than viral culture (34, 37). Inactivation of serum samples prior to nucleic acid amplification testing may be performed to increase the safety of specimen handling. In many countries of endemicity, molecular testing for CCHFV is commonly performed in BSL2 and BSL3 facilities (25). While many nucleic acid amplification tests are specific for CCHFV detection, several multiplex assays are available that are capable of detecting other viral hemorrhagic fever viruses (Table 1), which may be useful if the geographic region where infection was suspected to have been acquired is endemic for or the patient has risk factors for exposure to other hemorrhagic fever viruses. Nucleic acid amplification testing to date has focused on the detection of viral RNA in blood samples, although CCHFV RNA has, in some instances, been reported in saliva and urine samples (59).

Many RT-PCR assays provide the capability to quantify the level of CCHFV present. Quantification of the viral load may be useful for disease prognosis, as viral loads among survivors tend to be lower than among cases with a fatal outcome and may correlate with symptom severity (32, 34, 52, 56, 60, 61). The detection of a viral load of $\geq 1 \times 10^9$ copies/ml is an indicator of poor prognosis; in one study, viral loads of $\geq 1 \times 10^9$ copies/ml predicted a fatal outcome, with 88.9% sensitivity and 92.6% specificity (60, 61). In cases where patients survive CCHFV infection, viral load is more likely to decrease over the course of illness than that among fatal cases (32).

The sensitivity of nucleic acid amplification testing for CCHFV is complicated by the wide range of genetic diversity. Some assays have been studied using only limited numbers of strains or patient samples from limited geographic regions; while these assays may be appropriate for diagnosis of CCHF in the regions where circulating strains were studied, infections due to other CCHFV clades may be missed. Assays that have been studied against many or all CCHFV genetic clades are more appropriate for use across a broader geographic range and when the region of suspected acquisition is unknown (Table 1). In an external quality control study of nucleic acid amplification tests involving 44 laboratories offering CCHFV diagnostics worldwide, only 57% met criteria for optimal performance (all reference samples correctly identified) or acceptable performance (one missed positive reference sample) (29). The use of real-time

TABLE 1 Nucleic acid amplification tests for Crimean-Congo hemorrhagic fever virus

Authors	Assay type	Detects other viral hemorrhagic fever viruses?	Genomic target (segment)	No. of CCHFV strains and/or patient samples tested	Limit of detection	Yr published	Reference
Atkinson et al.	Real time RT-PCR	No	S	18 strains	5 RNA copies/reaction	2012	41
Bonney et al.	Isothermal recombinase polymerase amplification	No	S	12 strains; human and tick samples from Tajikistan	50–500 copies	2017	7
Brinkmann et al.	Multiplex amplification followed by next-generation sequencing	Yes	L	Patient samples from Turkey	Unknown	2017	42
Burt et al.	RT-PCR	No	S	Patient samples from southern Africa	Unknown	1998	37
Das et al.	Multiplex RT-PCR combined with universal array	Yes	S	3 strains	190 RNA copies/ml	2015	43
Drosten et al.	Real-time RT-PCR	Yes	S	Patient sample from Kosovo	2,779 genome equivalent/ml	2002	44
Duh et al.	One-step real-time RT-PCR	No	S	Patient samples from Kosovo	30 PFU/ml	2006	9
Fajfr et al.	Real-time RT-PCR	Yes	L	1 strain	33–100 fg/µl	2014	45
Filippone et al.	RT-PCR combined with a DNA microarray	Yes	L	4 strains; patient samples from the Balkans and Middle East	10^5–10^6 PFU/ml amplified cDNA	2013	46
Garrison et al.	Real-time RT-PCR	No	S	18 strains; patient samples from Uzbekistan	11.8 copies	2007	11
Ibrahim et al.	Real-time RT-PCR	No	S	1 strain	5 PFU	2011	47
Jaaskelainen et al.	Real-time RT-PCR	No	S	8 strains; patient samples from Turkey	11 genomes/reaction	2014	48
Kamboj et al.	Real-time RT-PCR	No	S	Animal and tick samples from India	7.6 copies	2014	49
Koehler et al.	Real-time RT-PCR	No	S	16 strains	256 PFU/ml	2018	50
Osman et al.	RT-LAMP[a]	No	S	Patient samples from Sudan	10 fg (naked eye turbidity), 0.1 fg (agarose gel electrophoresis)	2013	51
Papa et al.	Real-time RT-PCR	No	S	Patient samples from Albania	Unknown	2007	52
Sas et al.	One-step real-time RT-PCR	No	S	4 strains	2 copies/µl	2018	53
Schwarz et al.	Real-time RT-PCR	No	S	Patient samples from United Arab Emirates	Unknown	1996	54
Wölfel et al.	One-step RT-PCR combined with a DNA macroarray	No	S	18 strains; patient samples from Iran, Namibia, Pakistan, and South Africa	6.3 genome copies/reaction	2009	55
Wölfel et al.	Real-time RT-PCR	No	S	12 strains; patient samples from Iran, Pakistan, and South Africa	1,164 copies/ml	2007	56
Yapar et al.	One-step real-time RT-PCR	No	S	Patient samples from East Anatolia	10^2 genome equivalents/ml	2005	57
Zahraei et al.	One-step real-time RT-PCR	No	S	Patient samples from Iran	20 RNA copies/reaction	2016	58

[a]LAMP, loop-mediated isothermal amplification.

RT-PCR assays, real-time and conventional RT-PCR assays, and real-time and nested RT-PCR assays provided correct results in over 80% of reference samples, while the use of conventional RT-PCR or nested RT-PCR alone produced correct results in only 66% and 46% of samples, respectively (29).

Viral antigen detection. Viral antigen testing may be assessed using an enzyme-linked immunosorbent assay (ELISA) (62, 63). In mice, viral antigen can be detected 2 to 3 days after infection, with a lower limit of detection of 2.0 $\log_{10}$ focus-forming unit (FFU) virus/ml (63). In a human case series, viral antigen was detectable in 64% of CCHF patients and was most frequently detected among patients with positive viral cultures in the first 5 days of illness (94% positive) but was not detectable after day 9 of illness in any patients who survived the infection, although it could be detected among fatal cases (63). Another human case series demonstrated that viral antigen was detectable from patients with positive RT-PCR and negative antibody results; however, the sensitivity of antigen testing decreased once cases developed detectable anti-CCHFV antibodies (62). A decrease in the reactive ELISA signal was noted when anti-CCHF IgG antibodies were added to known quantities of viral antigen, suggesting that patient-generated anti-CCHFV antibodies may interfere with antigen ELISA testing (62). Antigen testing has the advantage of producing timely results compared with viral culture and requires less specialized equipment than nucleic acid amplification testing. Inactivation of serum samples may be performed to decrease the risk of inadvertent CCHFV infection among laboratory personnel performing ELISA testing.

In addition to serum testing, immunohistochemistry can be used to detect CCHFV antigens in tissue specimens. CCHFV antigens were detected by immunohistochemistry in liver tissue from 10 of 12 deceased patients with CCHF (64). However, unless biopsy specimens are obtained from a patient as part of the diagnostic evaluation, immuno-histochemical tissue evaluation is unlikely to be beneficial for diagnosing CCHF infection in the acute setting, although it may assist in retrospective diagnosis among fatal cases.

CCHF SEROLOGICAL TESTING

Historical serological testing methods for anti-CCHFV antibodies included agar gel diffusion precipitation, complement fixation, hemagglutination inhibition, indirect hemagglutination, reversed passive hemagglutination, and neutralization tests (65–67). These methods were replaced by ELISA and immunofluorescence assays due to im-proved reliability and sensitivity compared with older techniques (10, 68, 69). Many in-house ELISAs have been described utilizing either heat-inactivated CCHFV prepared from suckling mouse brain or, more commonly, recombinant CCHFV nucleocapsid protein as the target antigen (10, 41, 70–78). Several commercial ELISA kits are available for research use, but none are approved for human clinical diagnostic testing. One commercially available test, the VectoCrimea-CHF ELISA (Vector-Best, Novosibirsk, Russia), demonstrated a sensitivity of 87.8% (95% confidence interval [CI], 78.6% to 96.9%) and specificity of 98.9% (95% CI, 96.7% to 100.0%) for IgM antibodies and a sensitivity of 80.4% (95% CI, 69.5% to 91.3%) and specificity of 100% for IgG antibodies when tested across samples from a wide geographic range (69). This sensitivity was lower than the sensitivity reported when patient samples from Russia were tested (29). A separate ELISA using nucleocapsid derived from the AP92 strain was unable to detect antibodies to two known positive serum samples from different geographic regions (70). These findings suggest that the sensitivity of ELISA for antibody detection may be affected by the genetic variability of CCHFV.

Indirect immunofluorescence assays using recombinant CCHFV nucleoproteins ex-pressed in HeLa cells for antibody detection are commercially available for research use only (68). External validation of one commercially available immunofluorescence test, the Crimean-Congo fever mosaic 2 immunofluorescence assay (IFA) (Euroimmun, Lu-bick, Germany), demonstrated a sensitivity of 93.9% (95% CI, 85.8% to 100.0%) and specificity of 100% for IgM antibody detection and sensitivity of 86.1% (95% CI, 74.8% to 97.4%) with a specificity of 100% of IgG antibody detection (69).

Alternative serological testing methods include lateral flow assays and the use of Luminex technology for anti-CCHFV antibody detection. To date, one lateral flow assay has been developed for the detection of IgM antibodies against CCHFV; however, it was not useful for disease screening due to poor sensitivity (39.7%) when assessed using CCHF patient samples from Iran (79). The future development of lateral flow assays would be ideal for providing rapid test results in a point-of-care manner; however, as with ELISA, lateral flow assays are potentially subject to limitations in detecting antibodies to all CCHFV strains due to genetic variability. A Luminex xMAP assay exists that is capable of detecting IgG antibodies to a wide array of hemorrhagic fever viruses, including CCHFV, but has not yet been tested against a wide variety of CCHFV strains (80).

Neutralizing antibodies to CCHF may be measured using pseudoplaque or plaque reduction neutralization tests or CCHF viral-like particles (81–83). Neutralization assays using viral-like particles may be performed using BSL2 precautions (82). Neutralizing antibody assays are not routinely used for CCHF diagnosis.

SUMMARY AND CONCLUSIONS

The diagnosis of CCHFV infection is complicated by the nonspecific nature of symptoms, variable diagnostic utility of assays at different stages of infection, and the biosafety requirements for handling potentially infectious specimens. Many of the currently available tests, such viral culture and nucleic acid amplification testing, require specialized equipment not widely available to frontline health care workers in countries of endemicity. While some CCHF diagnostic assays may perform well in localized geographic regions, validated pan-CCHFV detection assays capable of detecting strains found throughout the world are desirable, although the development of such assays has been complicated by the high degree of CCHFV genetic diversity. Specimens collected from suspected CCHF cases should be treated as potentially infectious, and the biosafety requirements for performing CCHF diagnostic assays should follow country-specific guidelines. Assays requiring live virus should always be performed using the maximum level of biosafety precautions available, while inactivation techniques may increase the safety of performing nucleic acid amplification or serological testing using BSL2 or BSL3 facilities.

In the first week after symptom onset, direct viral detection assays are the diagnostic method of choice for CCHF, as antibodies to CCHFV are frequently absent during this period. Viral antigen and nucleic acid amplification tests provide more rapid results than viral culture, although they may be subject to more limitations in their abilities to detect a wide variety of CCHFV strains. In the second week of illness and beyond, serological assays assessing IgG and IgM antibodies to CCHFV are more likely to reveal the diagnosis. However, some patients who progress to fatal outcomes do not develop detectable levels of anti-CCHFV antibodies and may be missed using antibody ELISA or immunofluorescence assays alone. The use of a combination of direct viral testing and serological testing in the second week of illness and beyond would minimize the risk of missed CCHF diagnosis.

The ideal diagnostic assay for CCHF would provide rapid results and could be performed with low technical requirements as a point-of-care test to facilitate early therapeutic intervention and appropriate infection control precautions to minimize the potential for nosocomial spread. Assays should minimize specimen handling, avoid the use of sharps, and minimize aerosol production to reduce the risk of inadvertent secondary infection among laboratory staff, especially if testing not performed in a high containment facility. The World Health Organization draft roadmap for CCHF research proposed in 2018 includes a goal of having "rapid, reliable, simple-to-use and easily accessible diagnostics by 2023" to reduce morbidity and mortality from CCHF (84). Additional future research directions should include maximizing the ability of CCHF assays to accurately detect infection across different viral clades and to facilitate accurate diagnosis at all stages of illness.

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