Background: Crimean-Congo haemorrhagic fever virus (CCHFV) is considered an emerging infectious disease threat in the European Union. Since 2000, the incidence and geographic range of confirmed CCHF cases have markedly increased, following changes in the distribution of its main vector, *Hyalomma* ticks. Aims: To review scientific literature and collect experts’ opinion to analyse relevant aspects of the laboratory management of human CCHF cases and any exposed contacts, as well as identify areas for advancement of international collaborative preparedness and laboratory response plans. Methods: We conducted a literature review on CCHF molecular diagnostics through an online search. Further, we obtained expert opinions on the key laboratory aspects of CCHF diagnosis. Consulted experts were members of two European projects, EMERGE (Efficient response to highly dangerous and emerging pathogens at EU level) and EVD-LabNet (Emerging Viral Diseases-Expert Laboratory Network).Results: Consensus was reached on relevant and controversial aspects of CCHF disease with implications for laboratory management of human CCHF cases, including biosafety, diagnostic algorithm and advice to improve lab capabilities. Knowledge on the diffusion of CCHF can be obtained by promoting syndromic approach to infectious diseases diagnosis and by including CCHFV infection in the diagnostic algorithm of severe fevers of unknown origin. Conclusion: No effective vaccine and/or therapeutics are available at present so outbreak response relies on rapid identification and appropriate infection control measures. Frontline hospitals and reference laboratories have a crucial role in the response to a CCHF outbreak, which should integrate laboratory, clinical and public health responses.

Introduction

Crimean-Congo haemorrhagic fever virus (CCHFV) is a tick-borne pathogen that causes a frequently lethal disease in humans and is considered to be a major
Crimean-Congo haemorrhagic fever virus strains with complete S segment available as at 5 December 2017, were collected from GenBank database and clustered at 100% with CD-HIT v4.6. Sequences were aligned with MAFFT v7.123b. Phylogenetic analysis were performed using RAxML v8.2.10 with GTRGAMMA model and 1,000 bootstrap inferences. For graphical exemplification, only representative sequences in each clade for each country were selected and reported in the phylogenetic tree.

Branches owing to different clades are presented in the following colours: Africa: clade I (red), clade II (light green) and clade III (brown); Asia: clade IV (dark green); Europe: clade V (purple), clade VI (light blue) and clade VII (blue). For each strain we reported GenBank ID, isolate ID, host, country and collection date, if present. Bootstrap values are shown for each clade.
ecological investigation, as well as the absence of effective prophylaxis and treatment render CCHFV a pathogen with outbreak potential [4].

Since 2000, the incidence and geographic range of CCHF cases have markedly increased [5,6] following an expanding distribution of its main vector, ticks of the genus Hyalomma, specifically the Hyalomma marginatum species [1,7,8]. In Turkey, nearly 900 new CCHF cases occur annually, with a total of 9,787 cases reported from 2002–15 [9]. CCHF is endemic in the Balkan region, in Kosovo, 228 cases were reported from 1995–2013 [10], In Bulgaria, over 1,500 cases have been reported from 1952 [11]. In the European region, cases of human infection have also been reported from Albania, Russian Federation, Georgia, Greece, and Ukraine [12]. Imported cases have been reported in France [13], United Kingdom [14], Greece [15] and Germany [16]. A detailed review of other outbreaks has been recently published by Papa et al. [11].

Public health systems (including diagnostic laboratories) should be prepared to respond to the increased circulation of the virus in endemic EU countries, the potential for importation of human CCHF cases or the emergence of virus circulation in new areas e.g. Spain [17].

The objectives of this study were to amalgamate the expertise of two EU expert networks (i) EMERGE (Efficient response to highly dangerous and emerging pathogens at EU level) [18] and (ii) EVD-LabNet (Emerging Viral Diseases Laboratory Network) [19], in order to select and analyse the relevant and some of controversial aspects of CCHF disease diagnostics with implications for laboratory management of human CCHF cases and any exposed contacts.

**Methods**

We carried out an on line research of published paper related to CCHFV molecular detection methods. References were obtained by an online search in PubMed using an intentionally wide search-query to
ensure that a large number of papers was retrieved also for a rare disease such as CCHF.

The query produced a large number of papers, 20% of them were discarded after a narrative review, as they did not contain a detailed description of the detection methods employed including the nucleotide sequences of primers and/or probes. The search was done by one author and the results discussed among the authors. Papers related on non-previously retrieved molecular detection methods or to others relevant aspects discussed in this report have been directly provided by experts. For phylogenetic analysis all available CCHF virus genomes by 5 December 2017 were retrieved from GenBank (https://www.ncbi.nlm.nih.gov/nucleotide), using ‘txid1980519(Organism)’ as term of query. All analyses have been focused only on CCHFV S-segment, because it resulted as the most conserved gene across CCHFVs [8,20] and also because mostly all retrieved molecular methods has S segment as target. CCHF virus strains with complete S segment were selected and clustered at 100% with CD-HIT v4.6. A total of 163 sequences available at 5 December 2017 were obtained and aligned with MAFFT v7.123b in local pair mode. Phylogenetic analysis were performed with RAxML v8.2.10 using GTRGAMMA model and 1000 bootstrap inferences.

A preliminary text was drafted and discussed among the experts by email and during EMERGE and EVD-LabNet networks’ 2017 and 2018 annual meetings. Most of the relevant and some of controversial aspects of CCHF disease with implications for laboratory management have been selected and analysed in the following sections. In the present paper, all the expressed opinions take into account both published data and personal experience of the experts.

Results

Crimean-Congo haemorrhagic fever virus clades distribution

CCHFV (family *Nairoviridae*, genus *Orthonairovirus*) is tick-borne and is maintained in a tick-vertebrate-tick cycle with *Hyalomma marginatum*, the main vector species in Europe. Given the wide distribution of this vector, CCHFV has been detected over a wide geographic range: Africa, Europe, Asia and the Middle East [5,21].

CCHFV is an enveloped, tri-partite, negative-sense, RNA virus. The large genome segment (L) encodes the RNA-dependent RNA polymerase (L protein), the medium segment (M) encodes the glycoproteins GN and GC, while the small segment (S) encodes the nucleocapsid protein (N).

Phylogenetic tree (Figure 1) was built, including only 65 of 163 representative strains with reported location of provenance either in GenBank records or in the associated papers. Taking into account similarity and geographic locations of the different viral lineages, seven genetic clades were identified: three prevalently diffused in Africa (clades I-III), three in Europe (clades V, VI and VII) and one in the south of Asia (clade IV).

Most of the isolates causing outbreaks in eastern Europe belong to clade V, whereas clade VI and VII include largely divergent strains isolated from ticks in Greece (including the prototype strain AP92) [21,22] and Russia (GenBank accession number KR814833 and KR814835).

Moreover, isolates belonging to the African clade III were collected from infected ticks in 2010 and 2014, and recently [23] a virus aligning to this clade was the cause of an outbreak in Spain [17].

For all strains analysed, the collection country was recorded and represented on the world map in Figure 2.

Transmission mode

Human infections are usually observed as single, sporadic cases when people in rural areas are bitten by ticks that have become infected by feeding on viraemic wild and domestic animals like hares, hedgehogs, horses, livestock and possibly birds [21]. The infection in animals is generally asymptomatic; at most, a mild fever may be noted.

In addition to tick exposure, CCHF infection can result from direct contact, especially through mucous membranes or skin wounds, with crushed infected ticks or the blood of infected animals (principally among shepherds, farmers, abattoir workers and veterinarians). Person-to-person transmission can also occur through contact with virus-containing bodily fluids of patients during the first 7–10 days of illness [21]. Unprotected contact with other bodily fluids like saliva or urine, may also represent a risk for humans [24]. Nosocomial transmission to healthcare workers, transmission among patients sharing the same room [25] and possible sexual transmission [26,27] have also been reported.

Relevance of viraemia

The typical duration of viraemia ranges from 1–9 days [28,29], and there is so far no evidence of detectable viraemia during the incubation period [30]. However, the positivity of CCHFV RNA in serum has been exceptionally reported up to 36 days from the onset of symptoms [31]. Studies investigating the presence and persistence of CCHFV in other body fluids are limited. Viral RNA was detectable up to 10 days and as late as 25 days after onset of symptoms in saliva [24] and urine [31] respectively, but no data on virus viability are available.

Viral load is the most important prognostic factor: a value of viraemia higher than 10^6 copies/mL is associated with fatal outcomes [32]. Viraemia decreases significantly over time in surviving patients, but remains persistently high in non-survivors [32,33].
| Assay                  | Reference          | Reference testing material | Declared sensitivity/ specificity | Position in CCHFV strain IbAr10200 | Type               | Name             | Sequence                          |
|-----------------------|--------------------|----------------------------|-----------------------------------|------------------------------------|--------------------|------------------|-----------------------------------|
| **Single round PCR**  |                    |                            |                                   |                                    |                    |                  |                                   |
|                       | Drosten 2002 [70]   | Human clinical samples     | 95% detection limit of 2,779 copies per mL of serum | 351–579                           | forward primer     | CCS              | ATGCA6GAACCATTAAATRCTG7GA          |
|                       |                    |                            |                                   |                                    | reverse primer     | CCAS1            | CTAACATATCTGACACATTTTC             |
|                       |                    |                            |                                   |                                    | additional reverse primer | CCAS2          | CTAATCAGTCAGACGACTCT               |
|                       | Deyde 2006 [71]    | Human and animal laboratory isolates | ND                               | 1–1,672                           | forward primer     | SF               | TCTCAAAGAACCATG7GCGG              |
|                       |                    |                            |                                   |                                    | reverse primer     | SR               | TCTCAAAGATATCTG7GCGG              |
|                       | Schwarz 1996 [72]  | Human serum samples        | ND                               | 135–670                           | forward primer     | F2               | TGGCA6CCTTCACAAAC                  |
|                       |                    |                            |                                   |                                    | reverse primer     | R2               | GACATCA6CATTTCACCAGG              |
|                       | Midilli 2007 [73]  | Human serum samples        | ND                               | 119–762                           | forward primer     | CC-515F          | AARG6AAATGAGATTGGA               |
|                       |                    |                            |                                   |                                    | reverse primer     | CC-13F           | GGA6AYTCTACAAACCTC               |
|                       | Midilli 2009 a [74]| Human serum samples        | ND                               | 170–751                           | forward primer     | Gre-F1           | AAGTG6CAGAGAGTTGAG               |
|                       |                    |                            |                                   |                                    | reverse primer     | Gre-R1           | TGCG6CAATGCGCACTCCG              |
|                       | Midilli 2009 b [74]| Human serum samples        | ND                               | 192–501                           | forward primer     | Eec-F2           | ATCAGATGAGGACATTGCAACC            |
|                       |                    |                            |                                   |                                    | reverse primer     | Eec-R2           | ACTCCC6GTACACTAAATG              |
|                       | Elata 2011 [75]    | Human serum samples        | ND                               | 249–700                           | forward primer     | CCHF1             | CTTAAAGGCCTGGAGTTG1              |
|                       |                    |                            |                                   |                                    | reverse primer     | CCHF2_5          | TG6GCTGAG6GCGATG6ATAT             |
|                       | Negredo 2017 [17]  | Human serum samples        | ND                               | 123–764                           | forward primer     | CrCon1-             | RWAAYGGRCTTTRGTGAAYACYTTCCAC      |
|                       |                    |                            |                                   |                                    | reverse primer     | CrCon1-          | TRGCAAGRCCGTTWCRACWAGWG            |
|                       |                    |                            |                                   |                                    | reverse primer     | CrCon2-           | ARTG6GARARGAYTWGGYTYC             |
|                       |                    |                            |                                   |                                    | reverse primer     | CrCon2-           | CYTTG6GRAAYTCTRCACCABTC           |

CCHFV: Crimean Congo haemorrhagic fever virus; LAMP: loop-mediated isothermal amplification; ND: not declared; PFU: Plaque Forming Units; RPA: recombinase polymerase amplification; RT: reverse transcription.

For each assay, the position of amplicon is reported to respect IbAr10200 (NCBI reference sequence NC_005302). Names of each primer and probe correspond to those reported in the reference.
| Assay | Reference | Reference testing material | Declared sensitivity/specificity | Position in CCHFV strain IbAr10200 | Primers and probes | Type | Name | Sequence |
|-------|-----------|---------------------------|---------------------------------|-----------------------------------|---------------------|------|------|----------|
|       |           |                           |                                 |                                   |                     |      |      |          |
| Yapar 2005 [76] | Human serum samples | Linear detection 107–102 copies/mL | 1,160–1,242 | Forward primer | CCREalP1 | CCRealP1 | TCTTYGCHGATGAYTCHTYC |
|        |           |                           |                                 | Reverse primer | CCREalP2 | GGAATGKTYCCRAAGCA |
|        |           |                           |                                 | Probe | ND | ACAS RACRTAVATGCAYCCYTG |
| Duh 2006 [77] | Human serum samples | Viral RNA was detected until 30 PFU/mL | 296–484 | Forward primer | CCHFL1 | CCRealP1 | GGGAATGKTYCCRAAGCA |
|        |           |                           |                                 | Reverse primer | CCHFD1 | GGGATKGTYCCRAAGCA |
|        |           |                           |                                 | Probe | ND | ACAS RACRTAVATGCAYCCYTG |
| Wolfel 2007 [40] | Human serum samples | Analytical sensitivity in concentrations ranging from 100,000–10 copies per mL | 1,068–1,248 | Forward primer | RWCF | CCRealP1 | CAAAGGGTACCAAAGAAATGAGAAGGC |
|        |           |                           |                                 | Reverse primer | RWCR | GCCCAGGGATGTTCCAAAGCAGAC |
|        |           |                           |                                 | Probe | ND | ACAS RACRTAVATGCAYCCYTG |
| Garrison 2007 [78] | Laboratory isolates | Limit of detection 10 copies/mL; from 1.18 × 106–11.8 gene copies were linear | 649–705 | Forward primer | CCHF | CCHF | CAAAGGGTACCAAAGAAATGAGAAGGC |
|        |           |                           |                                 | Reverse primer | CCHF | CCHF | CAAAGGGTACCAAAGAAATGAGAAGGC |
|        |           |                           |                                 | Probe | ND | ACAS RACRTAVATGCAYCCYTG |
| Wolfel 2009 [79] | Laboratory isolates and human serum samples | 95% detection limit of 540 copies/mL of serum, corresponding to 0.5 genome copies/reaction | 210–489 | Forward primer | CCA_for | CCA_for | GTGCCACCTGTGATGACAAAGGATGACCTTCATTC |
|        |           |                           |                                 | Reverse primer | CCA_rev | CCA_rev | GTGTTGCCATGACGGAAACCTAGTGC |
|        |           |                           |                                 | Probe | ND | ACAS RACRTAVATGCAYCCYTG |
|        |           |                           |                                 | Additional forward primer | CCB_for | CCB_for | GTGCCACCTGTGATGACAAAGGATGACCTTCATTC |
|        |           |                           |                                 | Additional reverse primer | CCB_rev | CCB_rev | GTGCCACCTGTGATGACAAAGGATGACCTTCATTC |
| Atkinson 2012 [80] | Laboratory isolates | Ranging from 5,010, down to 0.5 copies of 5 segment RNA per reaction | 1–122 | Forward primer | CCHFS1 | CCHFS1 | TCTTAAAGGAACAGGCC |
|        |           |                           |                                 | Reverse primer | CCHFS222 | CCHFS222 | CCCTTTGAACCTTCAAACC |
|        |           |                           |                                 | Probe | ND | ACATCGAAGGAAACATGCGGCTAGAAG |
| Jaaskelainen 2014 [81] | Laboratory isolates and human serum samples | Sensitivity 100%; specificity 97% | 460–584 | Forward primer | FOR | FOR | GGACATAGGTTCCGACTCA |
|        |           |                           |                                 | Reverse primer | REV-1 | REV-1 | TCCCTCAATGCTGACAGC |
|        |           |                           |                                 | Additional reverse primer | REV-2 | REV-2 | TCTGCAAGCATACTCTTACAGAC |
|        |           |                           |                                 | Additional probe | probe1 | probe1 | TGGCGAAGTGATGCTG |
|        |           |                           |                                 | Additional probe | probe2 | probe2 | CTGCAAGTACAAAGTGT |
|        |           |                           |                                 | Additional probe | probe3 | probe3 | TRAGCAACAAAGTCCT |

CCHFV: Crimean-Congo haemorrhagic fever virus; LAMP: loop-mediated isothermal amplification; ND: not declared; PFU: Plaque Forming Units; RPA: recombinase polymerase amplification; RT: reverse transcription.

For each assay, the position of amplicon is reported to respect IbAr10200. Names of each primer and probe correspond to those reported in the reference.
| Assay             | Reference        | Reference testing material | Declared sensitivity/specificity | Position in CCHFV strain IbAr10200 | Primers and probes        |
|------------------|------------------|-----------------------------|----------------------------------|-----------------------------------|---------------------------|
|                  |                  |                             |                                  |                                   |                           |
| Real-time PCR    | Kamboj 2014 82   | Animal                      | Sensitivity from 7.6 x 10⁹ – 7.6 copies: specificity 100% | 32–137                            | Forward primer ND          |
|                  |                  |                             |                                  |                                   | Reverse primer ND          |
|                  |                  |                             |                                  |                                   | Probe ND                   |
|                  | Pang 2014 83     | Laboratory isolates         | Limit of detection from 133 RNA copies/PCR | 726–889                           | Forward primer ND          |
|                  |                  |                             |                                  |                                   | Reverse primer ND          |
|                  |                  |                             |                                  |                                   | Probe ND                   |
| Sybrgreen Real Time | Schneberger 2017 [84] | Laboratory isolates | ND                               | 86–244                            | Forward primer ND          |
|                  |                  |                             |                                  |                                   | Reverse primer ND          |
| LAMP             | Osman 2013 85    | Human serum samples         | Sensitivity in detecting ≥ 0.1 fg of viral RNA 100% | 1.063–1,266                      | Forward outer primer F3    |
|                  |                  |                             |                                  |                                   | Reverse outer primer B3    |
|                  |                  |                             |                                  |                                   | Inner primers              |
|                  |                  |                             |                                  |                                   | F1c                        |
|                  |                  |                             |                                  |                                   | F2                         |
|                  |                  |                             |                                  |                                   | B2                         |
|                  |                  |                             |                                  |                                   | B3c                        |
|                  |                  |                             |                                  |                                   | Loop primers               |
|                  |                  |                             |                                  |                                   | LF                         |
|                  |                  |                             |                                  |                                   | LB                         |
| RPA              | Bonney 2017 86   | Laboratory isolates         | Limit of detection from 5x10⁶ template copies – 50 copies | 8–158                             | Forward primer ND          |
|                  |                  |                             |                                  |                                   | Reverse primer ND          |
|                  |                  |                             |                                  |                                   | Probe ND                   |

CCHFV: Crimean-Congo haemorrhagic fever virus; LAMP: loop-mediated isothermal amplification; ND: not declared; PFU: Plaque Forming Units; RPA: recombinase polymerase amplification; RT: reverse transcription.

For each assay, the position of amplicon is reported to respect IbAr10200 (NCBI reference sequence NC_005302). Names of each primer and probe correspond to those reported in the reference.
Antibody kinetics

All CCHFV genotypes belong to one serogroup [21]. Cross-reactivity between CCHFV and other nairoviruses infecting humans (Ervé virus [34]), has not been described, although monoclonal and polyclonal antibodies to the N protein of CCHFV were found to cross-react weakly with Dugbe virus N protein [35].

Nucleocapside-directed IgM antibodies have been identified as the initial serological marker during infection, becoming detectable in a median of 2–3 days after disease onset, followed by glycoprotein precursor (GPC) directed IgM (4–6 days) and IgG antibodies (5–6 days) [28]. In another report, CCHFV IgM was detectable from 4 days after the onset of disease for up to 4 months. The maximum level of antibody titres was usually reached in the second to third week of illness [1]. IgM titre typically declines to undetectable levels 4 months after the onset of symptoms [29].

IgG seroconversion occurs 1–2 days after the IgM response [28] and IgG antibody remains detectable for at least 5 years [29,36].

Antibody production against CCHFV is an important prognostic indicator for survival [37]. Patients with fatal outcome rarely develop measurable antibody responses (reviewed in [37]) and Saksida et al. observed a reverse correlation between viral load and antibody levels in fatal CCHF cases [33], indicating that an impaired immune response leads to uncontrolled replication of the virus. High levels of interleukin-10 (IL-10), an anti-inflammatory cytokine, were detected in patients with fatal outcomes and were lowest in patients with a moderate disease course [33]. It was hypothesised that CCHF could be the result of a delayed and downregulated immune response caused by IL-10, which leads to an increased replication and spread of CCHFV throughout the body [33].

Biosafety

CCHFV is classified as a risk group 4 pathogen. The virus is stable under wet conditions for 7 hours at 37°C, 11 days at 20°C and 15 days at 4°C [20]. Under dry conditions, it is stable for at least 90 min, but less than 24 hours.

However, there is an ongoing debate about the absolute requirement of biosafety levels 4 (BSL4) for handling the virus [38]. Many endemic countries need to work with the virus despite the absence of BSL4 infrastructure. Biosafety and biosecurity procedures are essential for the safe and appropriate management of specimens from suspected/confirmed CCHF patients. All laboratories should refer to national guidelines on the documents ‘CWA 15793:2011 Laboratory bio-risk management’ and ‘CWA 16393:2012 Laboratory bio-risk management - Guidelines for the implementation of CWA 15793:2008’ for a complete guide [39]. The European Check List for Laboratory Biorisk Management developed in the framework of the Joint Actions Quality Assurance Exercises and Networking on the Detection of Highly Infectious Pathogens (QUANDHIP) project can be helpful for the implementation and evaluation of biorisk management approaches [40].

Inactivation

Like all lipid-enveloped viruses, CCHFV can be readily inactivated by common fixatives such as 2% glutaraldehyde [41], formalin and paraformaldehyde; chlorine-based disinfectants, such as 1% sodium hypochlorite [41,42]; and other disinfectants, such as hydrogen peroxide and peracetic acid [43,44]. Physical inactivation is also effective, like high temperature (56°C for 30 min or 60°C for 15 min) [41], Ultraviolet (1,200 to 3,000 µW/cm²) or low pH (less than 6) [43,45]. The virus does not survive in matured meat (due to low pH) and is also inactivated in 40% ethanol within 2 min [20].

There is a general agreement among the consulted experts that a critical aspect for laboratory biosafety and operation is the proper and reliable inactivation of specimens before they can be removed from the high-level biocontainment environment for further diagnostic testing. Some of these inactivation methods include (i) chemical treatment (i.e. Buffer AVL or Buffer RLT, Qiagen, Hilden, Germany) + 100% ethanol, SDS, 0.5% Tween-20 (Thermofisher, Waltham, Massachusetts, USA) [46,47] or (ii) heat treatment plus riboflavin (vitamin B) [48].

It is opinion of the experts that further evaluation of inactivation procedures are needed, especially for their impact on other laboratory tests necessary for clinical evaluation and increased survival rates of patients. Although there is no direct evidence of its effects on CCHFV, it has been shown that Triton X-100 (Sigma-Aldrich, Saint Louis, Missouri, USA) can decrease the biohazard risk of performing laboratory tests on samples from patients infected with other haemorrhagic fever viruses (i.e. Ebola), without affecting the results of biochemical tests [49-52].

Transport of diagnostic samples

General guidelines for suspected viral haemorrhagic fever infections apply for the transport of diagnostic samples from CCHF-suspected cases; these are listed under guidelines as Category A, Infectious Substances Affecting Humans UN 2814 and must be transported in packaging that meets the United Nations class 6.2 specifications and complies with Packing Instruction P620 [53].

Additionally, as for other biological resources, the exchange of CCHF samples or virus strains needs to comply with the Nagoya protocol on access to genetic resources and the fair and equitable sharing of benefits arising from their utilisation, which regulates transnational exchange between countries [54].
Diagnosis

The choice of which CCHF detection assays should be used for diagnostics with maximum sensitivity and specificity depends on the stage of disease and the specimens available.

Laboratory diagnosis of a patient with a clinical history compatible with CCHF is generally performed during the acute phase of the disease by viral RNA (RT-PCR) detection in blood [21]. In addition to blood (serum, plasma or whole blood), other possible specimens for molecular detection are saliva, urine [24,32] or post mortem biopsy of the liver and bodily fluids (including semen, for infection control purposes) [26,27].

The available data, limited to very few patients, do not allow detailed comparisons of the sensitivity of RT-PCR detection methods performed on different sample types and, in particular, of urine and saliva vs serum samples. In two of six infected patients reported in a review of CCHF cases in Kosovo* [31], viraemia was detectable up to 30 days after the onset of symptoms. In the same investigation, one patient’s urine was PCR-positive before the serum, and in another patient viruria continued longer than viraemia; however, more detailed studies on viruria are required. Further, both viruria and viraemia are detectable several days after the appearance of IgG response [31]. No chronological data are provided about detection of CCHFV RNA in saliva [24].

During the small outbreak in Spain involving two patients in 2016, positive RT-PCR results were obtained via saliva and vaginal swab, but they became negative when viraemia was still detectable [17]. Virus isolation was attempted from these samples, but was not successful (Maria Paz Sanchez-Seco, personal communication, EVD-LabNet 2nd annual meeting, Rotterdam October 2017).

Molecular detection

There is high genetic diversity within the different CCHFV strains (Figure 1), which consequently hampers the performance of molecular tests. As a result, a range of different methods employing varied primer/probe combinations have been developed and a truly universal assay has been difficult to devise. Table 1 lists published molecular assays retrieved by our PubMed search: two single round PCR, six nested PCR, 10 real-time PCR, one loop-mediated isothermal amplification (LAMP) and one recombinase polymerase amplification (RPA). Indication on reference testing materials and sensitivity/specificity of the tests are also reported, when declared.

Therefore there is an agreement of experts that is advisable to perform more than one test to avoid exclusive

---

Table 2: Commercial serological assays for Crimean-Congo haemorrhagic fever virus detection as at 5 December 2017

| Assay     | Detection mode | Diagnostic kit producers                                                                 | Comments/Target antigen                  |
|-----------|----------------|-----------------------------------------------------------------------------------------|------------------------------------------|
| IgG ELISA | Qualitative    | Vector-Best, Novosibirsk, Russia                                                        | Unknown                                  |
| IgM ELISA | Qualitative    | Euroimmun, Luebeck, Germany                                                            | CCHFV GPC and CCHFV N                    |
| IgG IFA   | Qualitative    | Crimea-Congo ELISA Kits, Diagen Biyoteknolojik Sistemleri A.Ş., Ankara, Turkey          | Unknown                                  |
| IgM ELISA | Quantitative   | Abbexa Ltd, Cambridge, United Kingdom                                                   | For research use only, not for diagnostic use. |
| Elisa     | Quantitative   | Alpha Diagnostic Intl. Inc., San Antonio, Texas, United States (US)                     | For research use only, not for diagnostic or therapeutic use. |
| IgM ELISA | Qualitative    | ELISA Kit, Antibody-Sunlong Biotech Co., Ltd, Hangzhou, Zhejiang, China                | Unknown                                  |
| IgG ELISA | Qualitative    | Creative Diagnostics, Shirley, New York, USA                                           | CCHFV NP                                |
| IgM ELISA | Quantitative   | Creative Diagnostics, Shirley, New York, USA                                           | CCHFV NP                                |

CCHFV: Crimean-Congo haemorrhagic fever virus; ELISA: enzyme-linked immunosorbent assay; IFA: immunofluorescent assay; USA: United States of America.

www.eurosurveillance.org
reliance on a single assay and a single target, taking into account the travel history and the geographic distribution of the different strains.

Serological assays
In published investigations, the methods employed for the detection of antibodies are indirect immunofluorescence assays (IFAs) and enzyme-linked immunosorbent assays (ELISAs) [55-57]. Several commercial kits are available (Table 2), but only the performance of Vector-Best CCHF ELISA and Euroimmun CCHF IFA have been tested in a collaborative study conducted by reference centres for CCHF laboratory diagnosis and surveillance in their respective countries [58]. The IgM sensitivity for ELISA and IFA assays were 87.8% (95% CI: 78.6–96.9) and 93.9% (95% CI: 85.8–100.0), respectively. For IgG assays, reported sensitivities were 80.4% (95% CI: 69.5–91.3) for ELISA and 86.1% (95% CI: 74.8–97.4) for IFA. The overall specificity was estimated at 100% for all the tests.

A CCHF seroneutralisation test is not normally performed for diagnostic purposes; it requires work with an infectious virus, necessitating a BSL4 laboratory, and is difficult to perform. However, reverse genetic approaches employing non-infectious reporter viruses have been described recently [59], enabling neutralisation to be performed at low containment.

Virus isolation
Viral isolation, i.e. from blood or organ for further characterisation or infectivity studies, is performed under BSL4 conditions on either LLC-MK2, Vero, BHK-21 or SW-13.4 cell lines and can be achieved in 2–10 days [60]. CCHFV generally produces no or little cytopathogenic effect and viral growth can be detected by IFA with specific monoclonal antibodies [41] or by molecular tests. When viral isolation on cell cultures fails, it can be attempted in new-born or immunodeficient mice.

### Laboratory diagnosis of CCHFV infection

There is no official, agreed-upon case definition for CCHF in the EU, though several case definitions adopted by EU countries were reviewed by the European Centre for Disease Prevention and Control (ECDC) [62,63]. According to a report published by ECDC in 2014 [60], most countries used, at least for surveillance purposes, the EU case definition established for Viral Haemorrhagic Fever [64].

Taking into account that laboratory screening is usually performed using molecular methods, we propose as expert opinion the following molecular diagnostic

---

**Box**

Criteria proposed for laboratory confirmation of a clinically suspected Crimean Congo haemorrhagic fever case

For laboratory confirmation of a clinical CCHF diagnosis, the expert group opinion is that a CCHFV infection is laboratory confirmed when at least one criteria in the Box is fulfilled.

- Detection by molecular tests of CCHFV RNA, in blood (whole blood, serum or plasma) or in other bodily fluids or tissues;
- Detection of CCHFV IgM or relevant (fourfold) increase in CCHFV IgG titres between two serologic samples (acute and convalescence phases);
- CCHFV isolation and/or detection of CCHF viral antigens in blood (whole blood, serum or plasma).

CCHFV: Crimean Congo haemorrhagic fever virus.

---

**Figure 3**

Algorithm for molecular diagnosis of Crimean-Congo haemorrhagic fever acute infection based on expert opinion

- Blood (Other additional sample)
- Molecular test
  - Negative
  - Positive
- Symptom onset
  - 0–3 days
  - 3 days

- Sequencing
  - Negative
  - Positive
  - Sequencing

CCHFV: Crimean-Congo haemorrhagic fever virus.

- The preferred biological specimen is blood (serum, plasma or whole blood). Other possible biological specimens are saliva, urine [24,31] and post-mortem biopsy or bodily fluids (including semen, for infection control purposes [26,27]).

- It is preferable that at least two different targets are tested: The first molecular test should target the S segment, while the second assay should be performed in a different genome region to confirm the absence or presence of CCHF infection, in case of negative result.

- Sequencing is indicated for viral characterisation and cluster identification, as well as for the confirmation of first cases detected or of discordant results of molecular tests.

- The molecular test to be performed should take into account epidemiological data.

It is preferable that viral isolation is performed on samples collected during the first 5 days of infection, when the viraemia levels are high [58,61].

---

**Table 2**

| Organisms                  | Commercial kits available |
|----------------------------|---------------------------|
| Vector-Best CCHF ELISA     | Yes                       |
| Euroimmun CCHF IFA         | Yes                       |

---

www.eurosurveillance.org
algorithm for patients with suspected CCHF infection (Figure 3). However, it is advisable, especially when the molecular tests are negative, to perform also serological tests on all suspected patients.

Other relevant aspects related to management of CCHF patients as clinical manifestation and discharging criteria are reported in the Supplementary material.

Discussion

CCHF is an important global health threat, as underlined by its inclusion in the list of priority diseases in the WHO document ‘An R & D blueprint for action to prevent epidemics’ [65].

In Europe, two autochthonous cases in Spain in 2016 [17] and the observed risk of importation of travel-associated cases [66] reinforce the notion that public health systems must be ready to respond to a potential emergence of CCHF.

Prompt and accurate laboratory diagnosis during the first days of the disease is critical to improve patient management, guide infection control measures and reduce case fatality. Early detection of viral RNA in blood is considered the gold standard diagnostic approach in the acute phase of the disease [21]. While the CCHFV RNA RT-PCR diagnostic test is most commonly performed in specialised laboratories, where non-commercial diagnostic assays and related reference biological material are available, this capability is more limited in rural areas and small cities where the majority of reported cases have occurred [9]. The availability of simple to use, commercial diagnostic tests will increase the number of laboratories performing RT-PCR or a similar NA detection strategy. However, laboratory capacity does not automatically mean capability, and laboratories implementing such tests would benefit from external quality assessments (EQA) of their capability to detect CCHFV in clinical samples, including monitoring of the effects of any corrective actions taken. The difficulty of clinical laboratories in establishing the diagnosis a CCHFV infection underlines the need to perform confirmatory tests in reference laboratories for both positive and negative samples collected from patients suspected of CCHF infection. In addition, appropriate biosafety measures must be in place when performing molecular testing.

Proposed measures to improve CCHFV laboratory preparedness and response

Measures to improve CCHFV laboratory preparedness and response should include: improving molecular tests to overcome the lack of sensitivity due to the high variability of the CCHFV genome; providing a positive control panel for molecular diagnostics, including the different CCHFV genotypes (including for serology testing) in order to support the improvement of diagnostic capability of clinical laboratories; enrolling diagnostic laboratories appointed for CCHFV diagnostics regularly in EQA programmes; improvement of diagnostic algorithms building on clinical experiences; validation of procedures to inactivate infectivity of clinical samples; and establishing of an international biorepository for the collection and storing of clinical samples, with the aim of validating new diagnostic tests and supporting pathogenicity studies. Some of these activities, including the evaluation of laboratory capability [67] and EQA [68] in particular, have been performed within the framework of EMERGE and EVD-LabNet and earlier as part of EU-funded projects such as EuroNetP4 (Grant No. 20033214), EnP4Lab (Grant No. 2006208), QUANDHIP (Grant No. 20102102) and ENIVD (Framework Service Contract ref. no. ECDC/2008/011. Similar support of other collaborating EU projects, such as the European Virus Archive (EVAg), could meet these needs in the future.

Conclusions

There are knowledge gaps concerning the putative persistence of the CCHFV in various body compartments of survivors and the related consequences for infection transmission. Basic knowledge is needed to provide evidence to better inform hospital discharge guidelines and these issues need further research. Furthermore, a One Health approach is required for adequate public health preparedness for CCHF, and relevant measures should include vector and animal surveillance, focusing particularly on migratory birds [5,9,69]. Greater awareness of the circulation of CCHFV in vectors/animals in specific geographic areas is fundamental in order to alert public health systems. Information on the circulation of CCHFV can be obtained by a syndromic approach and by including CCHF testing in the diagnostic algorithm of severe febrile infectious diseases of unknown origin.

Until an effective vaccine and/or therapeutics have been developed, the CCHFV outbreak response will continue to rely on rapid identification and appropriate infection-control measures. Front-line hospitals, as well as reference laboratories, have a crucial role in the outbreak response, which should integrate laboratory, clinical and public health responses.

Note

*This designation is without prejudice to positions on status, and is in line with United Nations Security Council Resolution 1244/99 and the International Court of Justice Opinion on the Kosovo Declaration of Independence.

Acknowledgements

This work was supported by the Health programme 2014–2020, from the European Commission; EMERGE Joint Action grant number: 677066. INMI received ‘Ricerca Corrente, Linea 1, Patogeni ad alto impatto sociale, emergenti, tropicali, MDR, negletti’ grants from the Italian Ministry of Health. This work was supported by the European Centre for Disease Prevention and Control (ECDC) under the EVD-LabNet Framework contract ECDC/2016/002. This work was supported by the CCHVaccine project 2 ‘the European
Conflict of interest

None declared.

Authors’ contributions

BB data analysis, coordinating the activities and writing manuscript; GCEM data analysis and writing manuscript; CBR and ADC study coordinator, data analysis and writing manuscript. BB, CEML, TA, SB, IC, RG, RH, GK, CML, AM, AP, MPSS, AVS, HZ, CN, MRC, GI, CBR, and ADC contributed to the conception and design of work, the interpretation of data, the revision of the manuscript and the approval of the final version.

References

1. Mertens M, Schmidt K, Ozkul A, Groschup MH. The impact of Crimean-Congo hemorrhagic fever virus on public health. Antiviral Res. 2013;99(2):248-60. https://doi.org/10.1016/j.antiviral.2013.02.007 PMID: 23458713

2. Dresch J, Ahmed S, Ramadan S, Dresch G, Humoli M, Dedushaj I. Current situation of Crimean-Congo hemorrhagic fever in Southeastern Europe and neighboring countries: a public health risk for the European Union? Travel Med Infect Dis. 2016;14(2):81-91. https://doi.org/10.1016/j.tmaid.2016.03.012 PMID: 27044611

3. Yon L, Duff JP, Egyptians, Erdelyi K, Ferroglio E, Godfroid J, et al. Recent changes in infectious diseases in European wildlife. J Wildl Dis. 2018; (Forthcoming). PMID: 30284963

4. Nisii C, Grunow R, Brave A, Ippolito G, Jacob D, Jureen P, et al. Genomic Characterization of Crimean-Congo hemorrhagic fever virus: A review of published field and laboratory studies. Antiviral Res. 2017;144-159. https://doi.org/10.1016/j.antiviral.2017.05.010 PMID: 28579441

5. Papa A, Tsergouli K, Tsioka K, Mirazimi A. Crimean-Congo hemorrhagic fever virus in Kosovo. PLoS Negl Trop Dis. 2014;8(1):e2647. https://doi.org/10.1371/journal.pntd.0002647 PMID: 24632218

6. Papa A, Weber F, Hewson R, Weidmann M, Koksal I, et al. Second International Conference on Crimean-Congo Hemorrhagic Fever Virus in Hyalomma Tick from Spain, 2014. Vector Borne Zoonotic Dis. 2015;15(10):1239-48. https://doi.org/10.1089/vbz.2014.2190 PMID: 25529825

7. Papa A, Markatou F, Maltezou HC, Papadopoulou E, Terzi E, Ventouris S, et al. Crimean-Congo hemorrhagic fever in a Greek worker returning from Bulgaria, June 2018. Euro Surveill. 2018;23(35):180452. https://doi.org/10.2807/1560-7917.ES.2018.23.35.180452 PMID: 30180928

8. Lumley S, Atkinson B, Dowall S, Pitman J, Staplehurst AL. Health care response to CCHF in US soldier and nosocomial transmission to health care providers, Germany, 2009. Emerg Infect Dis. 2015;21(1):23-31. https://doi.org/10.3201/eid2101.141413 PMID: 25108534

9. Papa A, Markatou F, Maltezou HC, Papadopoulou E, Terzi E, Ventouris S, et al. Crimean-Congo hemorrhagic fever imported into the United Kingdom (ex Bulgaria), June 2014. Euro Surveill. 2014;19(30):20864. https://doi.org/10.2807/1560-7917.EuS2014.19.30.20864 PMID: 25108534

10. Norberg A, de la Calle-Prieto F, Palencia-Herrejón E, Mora-Rillo T. Molecular epidemiology of Crimean-Congo hemorrhagic fever in Turkey: Current status and future challenges. Antiviral Res. 2016;126:21-34. https://doi.org/10.1016/j.antiviral.2015.12.003 PMID: 26695860

11. Papa A, Chaligiannis I, Kontana N, Sourba T, Tsioka K, Tsatsaris A, et al. A novel AP92-like Crimean-Congo hemorrhagic fever virus strain, Greece. Ticks Tick Borne Dis. 2014;5(3):590-3. https://doi.org/10.3201/eid2004.140408 PMID: 24953797

12. Cajimat MNB, Rodriguez SE, Schuster IUE, Swetnam DM, Ksiazek TG, Habela MA. et al. Genomic Characterization of Crimean-Congo Hemorrhagic Fever Virus in Hyalomma Tick from Spain, 2014. Vector Borne Zoonotic Dis. 2015;17(10):714-9. https://doi.org/10.1089/vbz.2015.0177 PMID: 26972040

13. Bodur H, Akinci E, Ongürü P, Carahan A, Uyar Y, Tanriaci A, et al. Detection of Crimean-Congo hemorrhagic fever virus genome in saliva and urine. Int J Infect Dis. 2016;45:109-11. https://doi.org/10.1016/j.ijid.2016.04.018 PMID: 27096742

14. Ergönül O, Battal I. Potential sexual transmission of Crimean-Congo Hemorrhagic Fever Virus. Viruses. 2016;8(12):123-47. https://doi.org/10.3389/viru.2016.01234 PMID: 27967002

15. Ergönül O, Battal I. Potential sexual transmission of Crimean-Congo hemorrhagic fever infection. Jpn J Infect Dis. 2014;67(2):137-8. https://doi.org/10.7883/yoken.67.137 PMID: 24647261

16. Ergunay K, Kocak Tufan Z, Bulut C, Kinikli S, Demiroz AP, Ozkül A. Antibody responses and viral load in patients with Crimean-Congo hemorrhagic fever: a comprehensive analysis during the early stages of the infection. Diagn Microbiol Infect Dis. 2014;79(1):31-6. https://doi.org/10.1016/j.diagmicrobio.2013.12.015 PMID: 24630756

17. Ergönül O. Crimean-Congo hemorrhagic fever. Lancet Infect Dis. 2006;6(6):393-6. https://doi.org/10.1016/S1473-3099(06)70245-4

18. Tishkova FH, Belobrova EA, Valikhodzhaeva M, Atkinson B, Ergönül O. Crimean-Congo Hemorrhagic Fever Virus in Hyalomma Tick from Bulgaria, June 2014. Vector Borne Zoonotic Dis. 2012;12(9):800-4. https://doi.org/10.1089/vbz.2011.0776 PMID: 22925025
review. Travel Med Infect Dis. 2016;14(2):73-80. https://doi.
.org/10.1016/j.tmaid.2016.03.002 PMID: 26970396

67. Fernandez-Garcia MD, Negredo A, Papa A, Donoso-Manrique
O, Niedrig M, Zeller H, et al. European survey on laboratory
preparedness, response and diagnostic capacity for
Crimean-Congo haemorrhagic fever. 2012. Euro Surveill.
2014;19(26):20844. https://doi.org/10.2807/1560-7917.
ES2014.19.26.20844 PMID: 25051064

68. Escadafal C, Olschäger S, Avsiç-Zupanc T, Papa A,
Vanhovenweg M, Wölfel R, et al. First international external
quality assessment of molecular detection of Crimean-Congo
hemorrhagic fever virus. PLoS Negl Trop Dis. 2012;6(12):e1706.
https://doi.org/10.1371/journal.pntd.0001706 PMID: 22745842

69. Gale P, Stephenson B, Brouwer A, Martinez M, de la Torre A,
Bosch J, et al. Impact of climate change on risk of incursion of
Crimean-Congo haemorrhagic fever virus in livestock in Europe
through migratory birds. J Appl Microbiol. 2012;112(2):246-57.
https://doi.org/10.1111/j.1365-2672.2011.05203.x PMID:
2218269

70. Drosten C, Göttig S, Schilling S, Asper M, Panning M, Schmitz
H, et al. Rapid detection and quantification of RNA of Ebola
and Marburg viruses, Lassa virus, Crimean-Congo hemorrhagic
fever virus, Rift Valley fever virus, dengue virus, and yellow
fever virus by real-time reverse transcription-PCR. J Clin
Microbiol. 2002;40(7):2323-30. https://doi.org/10.1128/JCM.
40.7.2323-2330.2002 PMID: 12089242

71. Deyde VM, Krhistova ML, Rollin PE, Ksiazek TG, Nichol ST.
Crimean-Congo hemorrhagic fever virus genomics and
global diversity. J Virol. 2006;80(17):8814-2. https://doi.
.org/10.1128/JVI.00752-06 PMID: 16912331

72. Schwarz TF, Nsanze H, Longson M, Nitschko H, Gilch S,
Shurie H, et al. Polymerase chain reaction for diagnosis and
identification of distinct variants of Crimean-Congo hemorrhagic
fever virus in the United Arab Emirates. Am J Trop Med Hyg.
1996;55(2):190-6. https://doi.org/10.4269/ajtmh.1996.55.2.
PMID: 8780459

73. Midilli K, Gargili A, Ergonul O, Sengöz G, Ozturk R, Bakar
M, et al. Imported Crimean-Congo hemorrhagic fever cases
in Istanbul. BMC Infect Dis. 2007;7(1):54. https://doi.
.org/10.1186/1471-2334-7-54 PMID: 17553137

74. Midilli K, Gargili A, Ergonul O, Eveli M, Ergin S, Turan N, et al.
The first clinical case due to AP92 like strain of Crimean-Congo
Hemorrhagic Fever virus and a field survey. BMC Infect Dis.
2009;9(3):90. https://doi.org/10.1186/1471-2334-9-90
PMID: 19352551

75. Elata AT, Karsany MS, Elageb RM, Hussain MA, Eltom KH,
Elbashir MI, et al. A nosocomial transmission of crimean-congo
hemorrhagic fever to an attending physician in North Kordufan,
Sudan. Virol J. 2011;8(1):303. https://doi.org/10.1186/1743-
422X-8-303 PMID: 21672268

76. Yapor M, Aydogan H, Pasha A, Besirellioglu BA, Budor H,
 Başustaoglu AC, et al. Rapid and quantitative detection of
Crimean-Congo hemorrhagic fever virus by one-step real-time
reverse transcriptase-PCR. Jpn J Infect Dis. 2005;58(6):358-62.
PMID: 16377867

77. Duh D, Saksida A, Petrovec M, Dedushaj I, Avsic-Zupanc T.
Novel one-step real-time RT-PCR assay for rapid and specific
diagnosis of Crimean-Congo hemorrhagic fever encountered in
the Balkans. J Virol Methods. 2006;133(2):175-9. https://doi.
.org/10.1016/j.jviromet.2005.11.006 PMID: 16343650

78. Garrison AR, Alakbarova S, Kulesh DA, Shezmukhamedova
D, Khodjaev S, Endy TP, et al. Development of a TaqMan
mini-gene multiplex qPCR approach for detection and
identification of Crimean-Congo hemorrhagic fever virus.
Am J Trop Med Hyg. 2009;77(3):514-20. https://doi.org/10.4269/
ajtmh.2007.77.514 PMID: 17827370

79. Wölfel R, Pawska JT, Petersen N, Grobelna AA, Leman PA,
Hewson R, et al. Low-density macroarray for rapid detection and
identification of Crimean-Congo hemorrhagic fever virus.
J Clin Microbiol. 2009;47(4):1205-30. https://doi.org/10.1128/
JCM.01920-08 PMID: 19225100

80. Atkinson B, Chamberlain J, Logue CH, Cook N, Bruce C, Dowall SD, et al. Development of a real-time RT-PCR assay
for the detection of Crimean-Congo hemorrhagic fever virus.
Vector Borne Zoonotic Dis. 2012;12(9):786-93. https://doi.
.org/10.1089/vbz.2011.0770 PMID: 22217755

81. Jääskeläinen AJ, Kallio-Kokko H, Ozkul A, Bodur H, Korukruoglu
G, Mousavi M, et al. Development and evaluation of a one-step
RT-qPCR for detection of Crimean-Congo hemorrhagic fever
virus representing different genotypes. Vector Borne Zoonotic
Dis. 2014;14(12):870-2. https://doi.org/10.1089/vbz.2014.1577
PMID: 25514124

82. Kamboj A, Pateriya AK, Mishra A, Ranaware P, Kulkarni RD,
Raut AA. Novel molecular beacon probe-based real-time RT-
PCR assay for the detection of Crimean-Congo hemorrhagic
fever encountered in India. BioMed Res Int. 2012;2014(496219):4.
https://doi.org/10.1155/2014/496219 PMID: 24877102

83. Pang Z, Li A, Li J, Qu J, He C, Zhang S, et al. Comprehensive
multiplex one-step real-time TaqMan qRT-PCR assays for
detection and quantification of hemorrhagic fever viruses.
PLoS One. 2014;9(4):e95635. https://doi.org/10.1371/journal.
pntd.00095635 PMID: 24752452

84. Schneeberger PHH, Poither JF, Bühmann A, Duffy B, Beuret
C, Uztinger J, et al. Development and evaluation of a
bioinformatics approach for designing molecular assays for
viral detection. PLoS One. 2017;12(5):e017895. https://doi.
.org/10.1371/journal.pone.017895 PMID: 28542435

85. Osman HA, Eltom KH, Musa NO, Bilal NM, Elbashir MI,
Aradaib IE. Development and evaluation of loop-mediated
isothermal amplification assay for detection of Crimean-Congo
hemorrhagic fever virus in Sudan. J Virol Methods. 2013;190(1-
2):4-10. https://doi.org/10.1016/j.jviromet.2013.03.004 PMID:
23542058

86. Bonney LC, Watson RJ, Afrough B, Mullojonoova M,
Dzhuraeva V, Tishkova F, et al. A recombinase polymerase
amplification assay for rapid detection of Crimean-Congo
Haemorrhagic Fever Virus infection. PLoS Negl Trop Dis.
2017;11(10):e0006013. https://doi.org/10.1371/journal.
pntd.0006013 PMID: 29028804

License and copyright

This is an open-access article distributed under the terms of the Creative Commons Attribution (CC BY 4.0) Licence. You
may share and adapt the material, but must give appropriate
credit to the source, provide a link to the licence, and indi-
cate if changes were made.

This article is copyright of the authors or their affiliated in-
stitutions, 2019.