

HISTORY AND CLASSIFICATION OF AIGAI VIRUS (FORMERLY CRIMEAN– CONGO HAEMORRHAGIC FEVER VIRUS GENOTYPE VI)

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Abbreviations: AIGV, Aigai virus; CCHF, Crimean-Congo hemorrhagic fever; CCHFV, Crimean-Congo hemorrhagic fever virus; ELISA, enzyme linked immunosorbent assay; ICD, International Classification of Diseases; ICTV, International Committee on Taxonomy of Viruses; IFA, indirect immunofluorescence assay; IgG, immunoglobulin G; L, large segment; M, medium segment; N, nucleocapsid protein; N, nucleocapsid gene; RdRp, RNA-directed RNA polymerase; TaxoProp, taxonomic proposal; USSR, Union of Soviet Socialist Republics; WHO, World Health Organisation. One supplementary table is available with the online version of this article

ABSTRACT

Crimean–Congo haemorrhagic fever virus (CCHFV) is the medically most important member of the rapidly expanding bunyaviral family *Nairoviridae*. Traditionally, CCHFV isolates have been assigned to six distinct genotypes. Here, the International Committee on Taxonomy of Viruses (ICTV) *Nairoviridae* Study Group outlines the reasons for the recent decision to re-classify genogroup VI (aka Europe-2 or AP-92-like) as a distinct virus, Aigai virus (AIGV).

INTRODUCTION

In 1944–1945, a novel severe human tick-borne disease was described among military personnel stationed on the Crimean peninsula (then part of the Crimean Autonomous Soviet Socialist Republic of the USSR and today internationally considered part of Ukraine) [1–3]. This disease, first called acute infectious capillary toxicosis and then Crimean haemorrhagic fever, is now known to occur in numerous African and Eurasian countries [4–8]. Outbreaks occur infrequently, typically infect only very few individuals, and most cases are asymptomatic or mild (e.g. headache, myalgia, joint pain, fever and nausea with vomiting). However, the disease may present with a sudden onset and rapid deterioration to severe haemorrhage, organ shutdown and death (lethality 5–80%) [3, 9–14]. The etiologic agent of this disease, an arbovirus, was first isolated in 1956 from a sick boy in Belgian Congo (today Democratic Republic of the Congo) [15–17]. The later discovery that the Crimean and Congolese diseases were identical and caused by the same virus ('Congo virus' = 'Crimean haemorrhagic fever virus') [18–20] is reflected in today's official name of the disease, Crimean–Congo haemorrhagic fever (CCHF; WHO ICD-11:1D49), and that of its causative virus, Crimean–Congo haemorrhagic fever virus (CCHFV) [21, 22]. CCHFV has a tri-segmented negative-sense RNA genome and produces enveloped particles. It is classified as an orthonairovirus in the bunyaviral family *Nairoviridae* (Negarnaviricota: Polyploviricotina) [21, 22]. In nature, CCHFV replicates in ixodid ticks of numerous species. These ticks transmit the virus to birds, mammals (including humans), and reptiles via bite, sometimes followed by direct transmission from animal to animal in the absence of ticks [4–8, 23, 24]. Phylogenetic analyses of presumed CCHFV isolates collected across Africa and Eurasia indicated the circulation of at least six distinct CCHFV genotypes: I–III (endemic in Africa), IV (Asia), V (Eastern Europe/Europe 1) and VI (Greece/Europe two or AP-92-like). A seventh (African) genotype is under discussion [25–31].

Initial Crimean–Congo haemorrhagic fever virus AP-92 isolation and sequencing

Genogroup VI (Greece/Europe 2 or AP-92-like) was established with a virus isolated by veterinarian Orestis Papadopoulos (Ορέστης Παπαδόπουλος) and his team at Aristotle University of Thessaloniki (Αριστοτέλειο Πανεπιστήμιο Θεσσαλονίκης), Thessaloniki, Greece. The team collected ixodid ticks (*Rhipicephalus bursa* Canestrini & Fanzago, 1878) on 22 May 1975, from a flock of domestic goats [*Capra aegagrus hircus* (Linnaeus, 1758)], kept at the foothills of a mountain, about 200m above sea level, in Vergina [Βεργίνα; ancient Aigai (Αιγαί), geographic coordinates: 40°28'31.19' N, 22°18'29.40' E], Imathia Regional Unit (Περιφερειακή ενότητα Ημαθίας) in the Central Macedonia Region (Περιφέρεια Κεντρικής Μακεδονίας), Greece [32, 33]. These collections were performed as part of a longitudinal study that had started in Vergina in 1969 to investigate a severe and often lethal disease of domestic goat kids caused by tick-borne encephalitis virus ('Greek goat encephalitis virus'; *Flaviviridae*: *Flavivirus*: Tick-borne encephalitis virus) [34].

Intraperitoneal inoculation of newborn laboratory mice with tick pool/phosphate-buffered saline homogenate supernatant and intraperitoneal or intracerebral inoculation of adult laboratory mice with supernatants did not cause any apparent phenotype. However, intracerebral inoculation of

newborn mice resulted in lethal disease. The etiologic agent was identified as CCHFV by complement fixation and immunodiffusion tests using mouse brain antigen preparations and reference anti-arbovirus antisera (hyperimmune mouse ascitic fluids). Antisera against 'Congo virus' and 'Crimean haemorrhagic fever virus'—provided by the Yale Arbovirus Research Unit, New Haven, Connecticut, USA, and the Institute of Poliomyelitis and Viral Encephalitides of the USSR Academy of Medical Sciences (Институт полиомиелита и вирусных энцефалитов АМН СССР), Moscow, USSR, respectively—reacted with the brain antigens. The novel isolate was named 'AP-92' [32, 33] after Αριθμός Πρωτοκόλλου-92 (Arithmós Protokóllou-92/Protocol Number 92) (Orestis Papadopoulos, personal communication with A.P.). Serum-precipitating antibodies against AP-92 were detected in 32.9% (139) of 422 screened goats and 11.6% (34) of 294 screened sheep but, in contrast to 'Congo virus' and 'Crimean haemorrhagic fever virus', no evidence suggested that AP-92 is a human pathogen [32, 33].

In 1980–1981, anti-CCHFV antibodies in humans were detected for the first time in Greece. Using indirect immunofluorescence assay (IFA) and haemagglutination-inhibition assays on sera from 65 residents of Imathia Regional Unit, four people were identified to possibly have had contact with CCHFV. The antigen used for these assays was derived from the CCHFV type virus, IbAr10200 (genotype III). Importantly, none of the four people that tested positive recalled any symptoms or clinical signs typical of severe CCHF [35]. In 1981–1988, a wider immunofluorescence assay survey was conducted using IbAR10200 antigen and sera obtained from 3388 individuals (mainly farmers, woodcutters and shepherds) living in 25 of 54 Greek counties. The results indicated an overall anti-CCHFV antibody seroprevalence of in 1.1% (37 of 3388 individuals) in Greece, with highest rates detected in Pella Regional Unit (Περιφερειακή ενότητα Πέλλα: 9.6%), Imathia Regional Unit (Περιφερειακή ενότητα Ημαθίας: 4.3%) and Karditsa Regional Unit (Περιφερειακή ενότητα Καρδίτσας: 6.2%) [36]. These and other serosurvey results [37] suggested that CCHFV, or a distinct but closely related virus, was/is indeed endemic in Greece.

Following the determination of the AP-92 nucleocapsid (N) gene sequence [encoded by the small (S) segment of the virus genome], an ELISA system was developed using AP-92 nucleocapsid (N) protein and three AP-92 N-derived peptides. There was no cross-reactivity with antibodies to non-CCHF orthonairoviruses, further supporting the notion that AP-92 is a strain of CCHFV [38]. Further characterization of AP-92 became possible after its complete genome sequence was reported in 2006. Comparison of this sequence with the genome sequences of numerous CCHFV isolates confirmed the overall clustering of AP-92 with CCHFV but also established AP-92 as a distinct clade (genotype VI) that is distantly related to all other CCHFV isolates [28]. In-depth analysis of the AP-92 glycoprotein precursor GPC [encoded by the medium (M) segment of the virus genome] further corroborated the special status of AP-92. In particular, the mucin-like domain in the amino-terminal part of AP-92 GPC and the so-called P38 region differ significantly from the orthologous sequences of CCHFV isolates [39]. Yet, until that time, there had been no evidence of CCHF in Greece.

The first CCHF case in Greece occurred in 2008, when a 46-year-old woman died in a hospital in Alexandroupoli (Αλεξανδρούπολη), in Eastern Macedonia and Thrace Region (Περιφέρεια Ανατολικής Μακεδονίας και Θράκης). The woman likely became infected after a tick bite, acquired during agricultural activities in Komotini (Κομοτηνή), close to the region's border to Bulgaria [40]. However, sequencing revealed the etiologic agent to belong to genotype V, closely related to Bulgarian CCHFV isolates, rather than to genotype VI [40, 41]. Since then, no further CCHF cases have been reported in Greece, with the exception of an imported case in a Greek worker returning from Bulgaria. Once again, the etiologic agent was identified as a genotype V CCHFV [42].

Further studies on AP-92

Since the case of CCHF in Greece in 2008, numerous seroprevalence studies have been conducted in that country to further define the risk of CCHFV infection in mammals [43], including humans [44–50]. Serum samples were collected together with questionnaires for study participants inquiring about demographic and epidemiological factors. A serological survey conducted from November 2008 until April 2009 in northeastern Greece indicated the presence of anti-CCHFV immunoglobulin G (IgG) in 3.1% (37 of 1,178) of people living in Eastern Macedonia and Thrace [47], whereas a country-wide study indicated a general anti-CCHFV antibody prevalence of 4.2% [44]. However, great differences were seen among regions, with the highest seroprevalence rates measured in mountainous areas among people reporting previous tick bites and in particular among people engaged in agricultural activities and animal slaughter [44, 46, 48–50]. All studies concluded that the high seroprevalence in specific regions in the absence of CCHF is likely due to either an avirulent CCHFV lineage or to endemicity of a non-CCHF orthonairovirus.

In addition to seroprevalence studies, ticks were surveyed for orthonairovirus infection. A virus was discovered in *R. bursa* ticks collected from sheep in the Western Macedonia Region. This virus differs from AP-92 by 9.7% at the nucleotide level but still clearly belongs to CCHFV genotype VI [51]. A country-wide study performed from 2012 until 2014 on 2000 ticks collected from livestock indicated a CCHFV infection prevalence of 2.8% (36 of 1290 tick pools with one–five ticks/pool). The number of collected *Hyalomma marginatum* Koch, 1844 ticks, the principal vector of CCHFV, was low with 0.5% of ticks. All specimens tested negative for CCHFV S segment sequence fragments of genotype V viruses were detected exclusively in kennel ticks [*Rhipicephalus sanguineus* (Latreille, 1806) *sensu lato*], whereas most AP-92-like sequences were found once again in *R. bursa* ticks [52]. In addition, an AP-92-like virus ('Pentalofos-Greece-2015') was isolated in a culture of the grivet epithelial kidney cell line Vero E6 with a pool of two adult *R. bursa* ticks removed from a domestic goat in Pentalofos (Πεντάλοφος), Thessaloniki Regional Unit. Anti-CCHFV IgG antibodies were detected in 8 of 19 goats of the index goat farm. This isolation enabled the sequencing a second complete AP-92-like genome, which further confirmed that AP-92-like viruses most strongly diverge from genotype I–V viruses in the M segment [53]. As of today, AP-92-like viruses have been found in *Rhipicephalus* spp. ticks in three Balkan countries other than Greece, namely Albania, Bulgaria and Kosovo [54–57]; in *H. marginatum*, *H. scupense* Schulze, 1919, and *R. bursa* ticks collected from cattle in Edirne and Kırklareli provinces, West Marmara Region (Batı Marmara Bölgesi) [58, 59]; in *H. marginatum*, *R. bursa* and *R. sanguineus sensu lato* ticks collected in Bayburt and Van Provinces in Northeast Anatolia Region (Kuzeydoğu Bölgesi) and in Mersin Province, Mediterranean Region (Akdeniz Bölgesi), Turkey [58, 60]; and in *Hyalomma aegyptium* Linnaeus, 1758 ticks collected from Greek tortoises (*Testudo graeca* Linnaeus, 1758) near Aflou (أفلو), Laghouat Province (الأغواط والية), Algeria [61]

AP-92 as the cause of human disease

In 2007 and in 2015, AP-92-like viruses were implicated in human CCHF-like disease for the first time [62, 63]. In 2007, 3 days after a tick bite, a 6-year-old boy with fever, malaise and loss of appetite was admitted to a hospital in Istanbul, Turkey. The child was hospitalized for 10 days and gradually improved. PCR and IgM and IgG ELISA tests were positive for CCHFV infection, and sequencing revealed the presence of an AP-92-like virus [62]. In 2015, a 60-year-old farmer from Kalārdasht (کالردشت), Māzandarān Province (مازندران استان), Iran, died of a severe CCHF-like disease caused by an AP-92-like virus [63].

Phylogenetic analyses consistently place AP-92-like viruses as a separate clade distant to CCHFV genotypes I–V/VII (Fig. 1). Phylogeographical reconstruction suggests that a common ancestor of

CCHFV genotypes I–V likely circulated in Africa and then spread to Asia in the 15th century CE. This virus then entered Europe on at least two occasions: first, in the early 1800s, when a still-circulating but less- or non-pathogenic virus emerged in what are now Greece and Turkey (genotype VI, ‘AP-92-like’) and, second, in the early 1900s, when a more virulent virus began to spread in eastern Europe (genotype V) [64]. Recent taxonomic analyses of the entire family *Nairoviridae* using pairwise amino acid sequence identity comparisons of the nairovirid RNA-directed RNA polymerase (RdRp) sequences, supported the separation of genotype VI (‘AP-92-like’) viruses from the CCHFV clade using the now generally established nairovirid species demarcation criteria of criteria of <93% identity in the amino acid sequence of the RdRp. Consequently, a taxonomic proposal (TaxoProp) by the International Committee on Taxonomy of Viruses (ICTV) *Nairoviridae* Study Group to reclassify AP-92-like viruses in a species separate from Crimean-Congo hemorrhagic fever virus [65] was accepted by the ICTV in early 2021. Accordingly, the new species Congoid orthonairovirus was established to harbour Aigai virus (AIGV), named after the place of discovery of the original AP-92 viruses and now representing all AP-92-like viruses [22, 66]. The ICTV has recently mandated that all established species names be changed to a newly standardized binomial format [66, 67]. Thus, most recently, the ICTV *Nairoviridae* Study Group supported a taxonomic proposal recommending the renaming of the two species to ‘*Orthonairovirus haemorrhagiae*’ and ‘*Orthonairovirus parahaemorrhagiae*’, respectively, among the renaming of all other nairovirid species (Table S1, available in the online version of this article) [68].

CONCLUSIONS

In summary, CCHFV genotype VI (Europe 2 or AP-92-like) viruses are sufficiently distinct from viruses in genotypes I–V/ VII to justify their reclassification into a novel orthonairovirus species, currently proposed to be renamed ‘*Orthonairovirus parahaemorrhagiae*’, for a distinct virus now called Aigai virus (AIGV). AIGV’s primary hosts appear to be *R. bursa* ticks, which are broadly distributed in Southern Europe, Northern Africa, South Asia and Western Asia, infesting primarily cattle, goats and sheep. Occasionally, these ticks also feed on other mammals, including humans [69], possibly explaining the rarity of reported human AIGV infections. Based on the two human infections, AIGV is a human pathogen, indicating the need for robust surveillance systems, identification methods and medical countermeasures. Development of these tools is essential to knowing where viral spread is occurring, monitoring cases and preventing outbreaks. Based on the low number of studied human infections, and experimental studies primarily having been performed with serially passaged and hence potentially attenuated strains, it would be foolish to underestimate AIGV’s virulence in humans. Consequently, we recommend assessing AIGV as a potentially significant human pathogen until proven otherwise

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Fig1. Phylogenetic position of Aigai virus (former Crimean–Congo haemorrhagic fever virus genotype VI). The orthonairovirus phylogenetic tree (top) was constructed based on a concatenated mafft-alignment of the three proteins encoded by the three orthonairovirus genome segments [nucleoprotein (S segment), glycoprotein precursor (M segment), large protein (L segment)], using the E-INS-i algorithm. The phylogeny was inferred using IQtree and the LG+I+G4 substitution model (best-fit model chosen according to BIC), assigned to every gene separately. Aigai virus (AIGV), formerly Crimean–Congo haemorrhagic fever virus (CCHFV) genotype VI is shown in red. The CCHFV-/AIGV-specific gene phylogenies (bottom) were based on mafft-alignments using the E-INS-i algorithm and were inferred using IQtree and the LG+I+G4 substitution model (best-fit model chosen according to BIC). Numbers on branch nodes represent transfer bootstrap expectation values (1000 replicates). Values below 60 are not shown. AIGV is indicated by red asterisks.

