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# Combination of RT-PCR and proteomics for the identification of Crimean-Congo hemorrhagic fever virus in ticks

Isabel G. Fernández de Mera<sup>a,1,\*</sup>, Ilias Chaligiannis<sup>b,1</sup>,  
Angélica Hernández-Jarguín<sup>a,1</sup>, Margarita Villar<sup>a</sup>, Lourdes Mateos-Hernández<sup>a</sup>,  
Anna Papa<sup>b</sup>, Smaragda Sotiraki<sup>c</sup>, Francisco Ruiz-Fons<sup>a</sup>, Alejandro Cabezas-Cruz<sup>d,e</sup>,  
Christian Gortázar<sup>a</sup>, José de la Fuente<sup>a,f,\*</sup>

<sup>a</sup> SaBio. Instituto de Investigación de Recursos Cinegéticos, IREC-CSIC-UCLM-JCCM, 13005 Ciudad Real, Spain

<sup>b</sup> Department of Microbiology, Medical School, Aristotle University of Thessaloniki, 54124, Thessaloniki, Greece

<sup>c</sup> Veterinary Research Institute, Hellenic Agricultural Organisation-Demeter, NAGREF Campus, 57001 Themi, Thessaloniki, Greece

<sup>d</sup> Institute of Parasitology, Biology Center of the Academy of Sciences of the Czech Republic, 37005 České Budějovice, Czech Republic

<sup>e</sup> Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic

<sup>f</sup> Department of Veterinary Pathobiology, Center for Veterinary Health Sciences, Oklahoma State University, Stillwater, Oklahoma, USA

\* Corresponding authors at: Instituto de Investigación de Recursos Cinegéticos, IREC-CSIC-UCLM-JCCM, 13005 Ciudad Real, Spain.

E-mail addresses: [mariaisabel.garcia@uclm.es](mailto:mariaisabel.garcia@uclm.es) (I.G. Fernández de Mera), [jose\\_delafuente@yahoo.com](mailto:jose_delafuente@yahoo.com) (J. de la Fuente).

<sup>1</sup> The first three authors contributed equally to the work reported in this paper.

## Abstract

Crimean-Congo hemorrhagic fever (CCHF) is an emerging tick-borne zoonotic disease caused by the CCHF virus (CCHFV). In this study, an experimental approach combining RT-PCR and proteomics was used for the identification and characterization of CCHFV in 106 ticks from 7 species that were collected from small ruminants in Greece. The methodological approach included an initial

screening for CCHFV by RT-PCR followed by proteomics analysis of positive and control negative tick samples. This novel approach allowed the identification of CCHFV-positive ticks and provided additional information to corroborate the RT-PCR findings using a different approach. Two ticks, *Dermacentor marginatus* and *Haemaphysalis parva* collected from a goat and a sheep, respectively were positive for CCHFV. The sequences for CCHFV RNA segments S and L were characterized by RT-PCR and proteomics analysis of tick samples, respectively. These results showed the possibility of combining analyses at the RNA and protein levels using RT-PCR and proteomics for the characterization of CCHFV in ticks. The results supported that the CCHFV identified in ticks are genetic variants of the AP92 strain. Although the AP92-like strains probably do not represent a high risk of CCHF to the population, the circulation of genetically diverse CCHFV strains could potentially result in the appearance of novel viral genotypes with increased pathogenicity and fitness.

**Keywords:** Infectious disease, Public health, Veterinary science, Evolution, Genetics, Virology

## 1. Introduction

Crimean-Congo hemorrhagic fever (CCHF) is an emerging tick-borne zoonotic disease causing sporadic cases or outbreaks of severe illness in humans (Ergonul, 2006; Bente et al., 2013; Papa et al., 2015a; Martina et al., 2017). CCHF is caused by the CCHF virus (CCHFV), which is distributed across a large geographic area from western China to the Middle East and Southeastern Europe and throughout most of Africa (Ergonul, 2006; Bente et al., 2013).

CCHFV is maintained in vertical and horizontal transmission cycles involving ixodid ticks and a variety of wild and domestic vertebrates, which do not show signs of illness but serve as reservoirs (Bente et al., 2013). The maintenance of active foci of CCHFV in the field may depend on tick survival, requiring favorable climatic conditions and high numbers of suitable hosts for adult ticks (Estrada-Peña et al., 2013). The virus circulates in a number of tick genera, but *Hyalomma* species are considered the principal source of human infection (Bente et al., 2013; Papa et al., 2015a). Humans could also be infected by contact with body fluids from infected viremic animals or patients (Bente et al., 2013; Papa et al., 2015a). CCHFV is considered the most genetically diverse of the arboviruses because it shows differences among genotypes ranging from 20% for the viral S segment to 31% for the M segment nucleotide sequences (Bente et al., 2013). Furthermore, different genotypes can be found within the same geographic area, while closely related genotypes have been isolated in geographically distant regions (Bente et al., 2013). Phylogenetic analysis of CCHFV from different locations suggests that the virus dispersed a long time ago, possibly by ticks carried on migratory birds or

through the international livestock trade (Anagnostou and Papa, 2009; Bente et al., 2013; Papa et al., 2015a).

Currently, CCHFV is diagnosed by virus isolation, serology and molecular-based techniques. Virus isolation requires laboratory biosafety level (BSL-3/4), which is not available in many institutions. Serology is useful in epidemiological studies of infected hosts by detection of CCHFV-specific IgM or IgG antibodies, but detection of anti-CCHFV IgM in infected patients requires at least 3–5 days post infection, which may account for false negative results during early acute phase of infection (Aradaib et al., 2011). Molecular techniques based on RT-PCR are used for the amplification of viral genome segments (Midilli et al., 2009; Osman et al., 2013; Bente et al., 2013; Papa et al., 2014; 2015a). Most of the RT-PCR assays use a secondary nested amplification or nucleic acid hybridization to increase the sensitivity and to confirm the identity of the amplified PCR product (Aradaib et al., 2011; Osman et al., 2013).

However, despite virus amplification after multiplication in ticks feeding on a susceptible host (Dickson and Turell, 1992; Bente et al., 2013; de la Fuente et al., 2017), CCHFV identification in individual ticks may be difficult due to low infection levels and the presence of other Nairoviruses (Walker et al., 2015). In this regard, the proteomics approach using matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) would provide additional information to corroborate the RT-PCR findings using a different approach, and assist in the differentiation between different CCHFV genotypes and Nairoviruses (Singhal et al., 2015) and the characterization of tick-virus molecular interactions (Papa et al., 2017).

In this study, a novel approach using the combination of RT-PCR and MALDI-TOF MS was used for the molecular identification and characterization of CCHFV in ticks collected from domestic ruminants in endemic areas of Greece.

## 2. Materials and methods

Partially fed ticks from 7 species (N = 106) were collected in 10 Greek counties from sheep and goats, which are considered the most suitable indicator animals for the circulation of CCHFV (Schuster et al., 2016) (Fig. 1 and Table 1). Collected ticks were classified (Manilla, 1998) and frozen until dissection of internal organs for analysis. Ticks were divided in two similar vertical halves to dissect internal organs for RNA and protein studies. Total RNA was extracted from one half ticks using TriReagent (Sigma, St. Louis, MO, USA) and following manufacturer recommendations. CCHFV RNA was amplified using a nested RT-PCR targeting virus S segment as previously described (Midilli et al., 2009). The amplicons were cloned and at least 3 clones were sequenced for each amplicon.



**Fig. 1.** Tick sampling. Map of Greece showing counties where ticks were collected from sheep and goats. The sites where CCHFV-positive ticks were collected are shown in green.

For proteomics analysis, internal tissues were dissected from the another half of CCHV-infected ( $n = 2$ ) and uninfected ( $n = 4$ ) ticks, lysed with phosphate buffered saline (PBS) supplemented with 1% Triton X-100 and complete protease inhibitor cocktail (Roche, Basel, Switzerland), and homogenized by passing through a needle (27G). Samples were sonicated for 1 min in an ultrasonic cooled bath, followed by vortexing for 10 sec. After three cycles of sonication-vortex, tick lysates were centrifuged at  $200 \times g$  for 5 min to remove cell debris. The supernatants were collected and protein concentration was determined using the BCA Protein Assay (Life Technologies, Carlsbad, CA) with BSA as standard. Protein extracts (10  $\mu$ g) from each CCHV-infected and uninfected ticks were on-gel concentrated by SDS-PAGE and trypsin digested as described previously (Villar et al., 2015). The desalted protein digest was resuspended in 0.1% formic acid and analyzed by RP-LC-MS/MS using an Easy-nLC II system coupled to an ion trap LTQ mass spectrometer (Thermo Scientific, Waltham, MA, USA). The peptides were concentrated (on-line) by reverse phase chromatography using a  $0.1 \times 20$  mm C18 RP precolumn (Thermo Scientific), and then separated using a  $0.075 \times 100$  mm C18 RP column (Thermo Scientific) operating at 0.3 ml/min. Peptides were eluted using a 60-min gradient from 5 to 40% solvent B (solvent A: 0.1%

**Table 1.** Tick species collected from sheep and goats in Greece and used in the study.

Tick species	N (female, male)	Tick hosts (N)	County (N)
<i>Dermacentor marginatus</i>	40 (20, 20)	sheep (17), goat (23)	Arta (3) Etoloakarnania (3) Evritania (6) Fokida (11) Ftiotida (1) Korinthos (14) <sup>†</sup> Preveza (2)
<i>Haemaphysalis parva</i>	24 (20, 4)	sheep (16), goat (8)	Arta (3) Etoloakarnania (1) Evritania (4) Fokida (9) <sup>†</sup> Ftiotida (2) Preveza (3) Thessaloniki (2)
<i>Haemaphysalis punctata</i>	1 (0, 1)	sheep (1)	Arta (1)
<i>Haemaphysalis sulcata</i>	22 (19, 3)	sheep (15), goat (7)	Arta (4) Etoloakarnania (2) Fokida (11) Preveza (4) Thessaloniki (1)
<i>Haemaphysalis</i> sp.	1 (1, 0)	sheep (1)	Ftiotida (1)
<i>Ixodes gibbosus</i>	13 (13, 0)	sheep (13)	Limnos (13)
<i>Rhipicephalus sanguineus</i>	4 (4, 0)	sheep (1), goat (3)	Limnos (1) Rodopi (3)
<i>Rhipicephalus bursa</i>	1 (0, 1)	goat (1)	Rodopi (1)

Ticks were collected from sheep and goats in Greece and identified to species level. <sup>†</sup>Tick samples positive for CCHFV by RT-PCR and sequence analysis of the S segment, and by proteomics analysis of the L segment.

formic acid in water, solvent B: 0,1% formic acid in acetonitrile). ESI ionization was done using a Fused-silica PicoTip Emitter ID 10 mm (New Objective, Woburn, MA, USA) interface. Peptides were detected in survey scans from 400 to 1600 amu (1 mscan), followed by fifteen data dependent MS/MS scans (Top 15), using an isolation width of 2 mass-to-charge ratio units, normalized collision energy of 35%, and dynamic exclusion applied during 30 sec periods. The MS/MS raw files were searched against a compiled database containing all sequences from Ixodida (81,241 entries in March 2016), Ruminantia (87,986 entries in March

2016) and Nairovirus (847 entries in March 2016) Uniprot taxonomies (<http://www.uniprot.org>) using the SEQUEST algorithm (Proteome Discoverer 1.4, Thermo Scientific). The following constraints were used for the searches: tryptic cleavage after Arg and Lys, up to two missed cleavage sites, and tolerances of 1 Da for precursor ions and 0.8 Da for MS/MS fragment ions and the searches were performed allowing optional Met oxidation and Cys carbamidomethylation. Data are available via PeptideAtlas with identifier PASS00907. A false discovery rate (FDR) < 0.01 was considered as condition for successful peptide assignments and at least 2 peptides per protein were used for protein identification.

### 3. Results and discussion

The results showed that 2 of the 106 sampled ticks were positive for CCHFV by RT-PCR (Table 1). The positive ticks corresponded to *Dermacentor marginatus* and *Haemaphysalis parva* collected from a goat and a sheep at Korinthos and Fokida counties, respectively (Fig. 1 and Table 1). These tick species were previously reported to be infected with CCHFV (Albayrak et al., 2010; Hekimoglu et al., 2012). The sequences of the S segment (GenBank accession numbers KU365757 and KU365758) were 99% identical between them at the nucleotide and amino acid levels. Furthermore, these sequences showed a 98% and 99–100% identity at the nucleotide and amino acid levels, respectively to CCHFV strain AP92 (DQ211638) previously isolated from a *Rhipicephalus bursa* tick. To confirm the identity of amplified and sequenced PCR products, a proteomics analysis was conducted using tissues from infected and control uninfected ticks. Tick- and host-derived proteins were identified in both infected and uninfected ticks, and were excluded from the analysis. Nairovirus-derived peptides corresponding to the L segment containing a putative RNA-dependent RNA polymerase (Bente et al., 2013), and unique to CCHFV were identified in infected *D. marginatus* and *H. parva* ticks (Table 2). The results confirmed CCHFV infection in these ticks with an alternative method and a different genome region (L segment) from that targeted by RT-PCR (S segment). The proteomics analysis was targeted at the L segment because this segment showed a higher peptide identification among different Nairoviruses in this study. The sequences for L segment peptides No. 1, 2 and 5 (Table 2) were 100% identical to the corresponding sequence of the prototype AP92 strain (ABB30012).

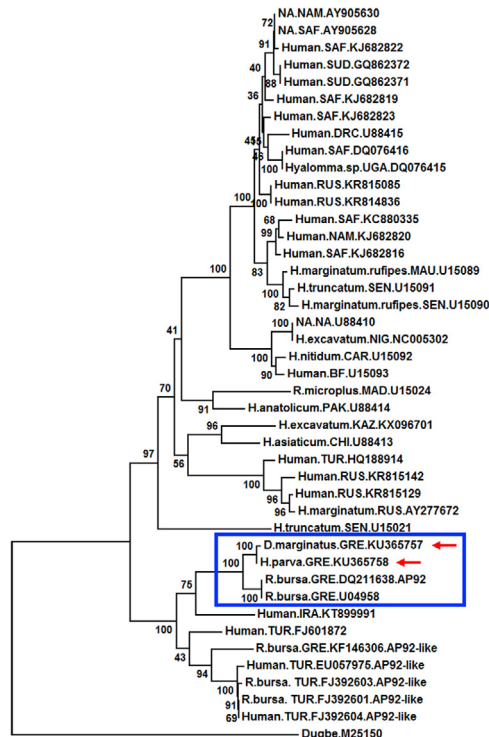
The results suggested that the CCHFV identified in the present study in ticks are genetic variants of the AP92 strain, but are closer to the old AP92 strain (98% nucleotide sequence identity) than to the novel AP92-like strain (91% nucleotide sequence identity) (Fig. 2). Greece is a Mediterranean country where currently only one CCHF case has been reported (Papa et al., 2015a). The absence of CCHF cases in Greece, together with the high seroprevalence in the human population, and the fact that one of the veterinarians who isolated the AP92 strain

**Table 2.** Nairovirus-derived peptides identified in infected ticks.

No.	Peptide sequence (sequence identity among CCHFV genotypes)	Sequence alignment	Nairovirus (GenBank accession number)
1	TELLNSLTLLHCFLKHAPSDAIMEVESK (100%)	<u>TELLNSLTLLHCFLKHAP</u> –SDAIMEVESK <u>TELLNSLCLLHCFLKHTS</u> –QDAIQEVESK <u>DQLYSSL</u> <u>SL</u> <u>LHCFFCHTL</u> –TSSVMEASSK <u>DDVVLNSIAL</u> <u>LHVFMHHAP</u> –KAAILEMNSK	CCHFV (Q6TFZ8) Dugbe virus (NP_690576) Hughes virus (AMT75407) Sakhalin virus (AMT75419)
2	IVFAKIGLSGNGYDFIWTTQMIANSNFNVCKR (97–100%)	<u>IVFAKIGLSGNGYDFIWTTQMIANSNFNVCK</u> <u>-VFAKLGLSGN</u> <u>NYDFIWTLQMIANSNFNVCK</u> <u>-VFAKMGLSDKSYDFIWTVQMIANSNFNVCK</u>	CCHFV (Q6TFZ8) Dugbe virus (NP_690576) Thiafora virus (ALD84355)
3	VLDCMFNCKLCVEISADTLILRPESKER (86–100%)	<u>VLDCMFNCKLCVEISADTLILRPESKER</u> <u>VLDCLFSCEV</u> <u>CEIESGIL</u> –LLKQKTQENSKTTLSLSR <u>VLHKIFNCKIAV</u> <u>SLDEGLLYLRPETRE</u> <u>VLDTF</u> <u>FCNVEV</u> <u>SLTSKV</u> –LYLLPEGSDDPNRVTLISKIR	CCHFV (Q6TFZ8) Dugbe virus (NP_690576) Hughes virus (AMT75407) Sakhalin virus (AMT75419)
4	RDDEELTNSSSLK (62–100%)	<u>RDDEELTNSSSLK</u>	CCHFV (Q6TFZ8)
5	FTWFQEVVLYGKICETFLRCCTEFNR (96–100%)	<u>FTWFQEVVLYGKICETFLRCCTEFNR</u> <u>FKWYQKL</u> <u>VYYGKICETFLQCCTEFTR</u> <u>FGWFQEVVLYSKICETFLRCCTEFNR</u> <u>FKWYQKL</u> <u>VLYGKICETFLQCCTEFRR</u> <u>FGWYQEL</u> <u>VLYSKICETFLRCCTEFTR</u> <u>FTWFQEVLLY</u> <u>SKICETFLRICTEFNR</u>	CCHFV (Q6TFZ8) Dera Ghazi Khan virus (AMT75389) Dugbe virus (NP_690576) Hughes virus (AMT75407) Sakhalin virus (AMT75419) Thiafora virus (ALD84355)
6	FMNIHAPELMPENCLFSSEEFNELIKLKK (74–100%)	<u>FMNIHAPELMPENCLFSSEEFNELIKLKK</u>	CCHFV (Q6TFZ8)

The identified Nairovirus-derived peptides corresponding to the L segment were aligned to viral amino acid sequences available at the GenBank using BLAST. Conserved amino acids when compared to the CCHFV sequence are shown underlined. Only virus sequences for which a hit was found after alignment are shown. Sequence identity among CCHFV genotypes (in parenthesis) was obtained after alignment with all available CCHFV sequences.





**Fig. 2.** Phylogenetic analysis of CCHFV. A phylogenetic tree was built using S gene nucleotide sequences from CCHFV. The CCHFV sequences obtained in this study are shown with red arrows. In the tree, the different isolates were annotated as “host.country.NCBI accession number”. The country code is Burkina Faso (BF), Central African Republic (CAR), China (CHI), Democratic Republic of Congo (DRC), Greece (GRE), Iran (IRA), Kazakhstan (KAZ), Mauritania (MAU), Madagascar (MAD), Namibia (NAM), Nigeria (NIG), Pakistan (PAK), Russia (RUS), Sudan (SUD), South Africa (SAF), Senegal (SEN), Turkey (TUR), Uganda (UGA), and NA (not available). The DQ211638 and U04958 represent the same isolate corresponding to the prototype AP92 strain. Nucleotide sequences were aligned using MAFFT version 7.0 (<http://mafft.cbrc.jp/alignment/software/>). Non-aligned regions were removed with Gblocks (version 0.91b) implemented in Phylogeny.fr. The final cured alignments contained 396 gap-free nucleotide positions. The best-fit model of the sequence evolution was selected based on Corrected Akaike Information Criterion (cAIC) and Bayesian Information Criterion (BIC) implemented in Molecular Evolutionary Genetics Analysis (MEGA) version 6.0 (<http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3840312/>). The Kimura-2 model, which had the lowest values of cAIC and BIC, was chosen for subsequent phylogenetic analyses. The Neighbor joining (NJ) method implemented in MEGA, was used to obtain the best tree topology. A proportion of Gamma distributed sites (+G, 0.24) was estimated in MEGA. Dugbe virus was used as outgroup. Reliability of internal branches was assessed using the bootstrapping method (1000 replicates). Graphical representation and editing of the phylogenetic tree was performed with MEGA.

demonstrated very high titres of anti-CCHFV antibodies, led to the suggestion that the AP92 strain is not pathogenic for humans (Papa et al., 2015b). Besides the prototype AP92 strain, a novel AP92-like strain (KF146306) has been recently detected in Greece in *R. bursa* collected from sheep in an area with 6% CCHFV seroprevalence (Papa et al., 2014). Currently, one human case with mild symptoms has been associated with the AP92-like strain in Turkey (Midilli et al., 2009), and

one fatal case due to the AP92-like strain was recently reported in Iran ([Salehi-Vaziri et al., 2016](#)).

These results showed the possibility of using this experimental approach for the identification and characterization of CCHFV in ticks, and confirmed the presence of genetically diverse CCHFV strains in different tick species collected from goats and sheep in Greece. The combination of RT-PCR with MALDI-TOF MS increases the possibility of characterizing CCHFV genetic variants circulating in different regions, and differentiating CCHFV from other Nairoviruses ([Table 2](#)). CCHFV shows a higher genetic diversity when compared to other arboviruses, a finding that has been linked to increased virulence and emergence in new geographic locations ([Xia et al., 2016](#)). These results also supported the increasing evidence that the circulation of the low or not pathogenic AP92-like strains probably does not represent a high risk of CCHF to the population. However, the circulation of genetically diverse CCHFV strains could potentially result in the appearance of novel viral genotypes with increased pathogenicity and fitness ([Bente et al., 2013](#)).

## Declarations

### Author contribution statement

Isabel G Fernandez de Mera, Ilias Chaligianis: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Angelica Hernández Jarguin, Lourdes Mateos-Hernández, Francisco Ruiz-Fons: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Margarita Villar: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Anna Papa, Jose de la Fuente: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Smaragda Sotiraki: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Alejandro Cabezas-Cruz: Analyzed and interpreted the data; Wrote the paper.

Christian Gortazar: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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## Competing interest statement

The authors declare no conflict of interest.

## Additional information

No additional information is available for this paper.

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