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Serological surveillance of Crimean-Congo hemorrhagic fever virus in Hungary and RNA interference as an effective method of inhibiting viral replication

PhD Thesis

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ABBREVIATIONS

ATP – Adenosine-5′-trifoszfát

B-PER – Bacterial Protein Extraction reagent

BSA – Bovine serum albumin

BSL-4 – Biosafety Level 4

CC50 – Cytotoxic concentration of the extracts leading to death in 50% of the cells

CCHF – Crimean-Congo hemorrhagic fever

CCHFV – Crimean-Congo hemorrhagic fever virus

CHF – Crimean hemorrhagic fever

DBA – Dot-blot assay

ddPCR – Droplet Digital PCR

DMEM – Dulbecco's modified eagle medium

ECL – Enhanced Chemiluminescence

FBS – Fetal bovine serum

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HRP – Horseradish peroxidase

IFA – Immunofluorescent assay

IgG – Immunoglobulin G

IPTG – Isopropyl D-1-thiogalactopyranoside

MVB – Multivesicular body

NCR – Non-coding region

NP – Nucleocapsid protein

PBS – Phosphate buffered saline

PCR – Polymerase chain reaction

PTGS – Post-transcriptional gene silencing

qRT-PCR – Quantitative reverse transcription-polymerase chain reaction

RdRp – RNA dependent RNA polymerase

RISC – RNA-Induced Silencing Complex

RNAi – RNA interference

RNP – Ribonucleoprotein

RT-ddPCR – Reverse transcription droplet digital polymerase chain reaction

SDS – Sodium dodecyl sulfate

siRNA – Small interfering RNA

TBS – Tris-buffered saline

TBS-T – Tris-buffered saline plus Tween-20 additive

TCID50 – Median tissue culture infectious dose

TGN – Trans-Golgi network

WB – Wester-blot

WHO – World Health Organization

1. INTRODUCTION

Zoonoses diseases or infections are naturally transmissible from animals (usually vertebrates) to humans. Zoonotic agents can be viruses, bacteria, fungi and parasites. It has been described by the World Health Organization (WHO) and Graham and colleagues [1] in which approximately 75% of new emerging human infectious diseases are defined as zoonoses. The best-known zoonoses include avian influenza, malaria, rabies, Ebola virus, yellow fever, Nipah and Hendra virus, SARS-Coronavirus, etc.

Over the past three decades, emerging and re-emerging zoonoses have appeared, partly as a result of increasing human dependence upon one another for animals and their products, and our close association with companion animals. Therefore, zoonoses are to be considered as the most critical risk factor regarding infectious diseases for human health and well-being [2].

Several factors can facilitate the appearance and reappearance of zoonotic infections and primarily are associated with changes in the environment and human activity. Climate change, the increasing intensity of agriculture and deforestation, the construction of dams and other irrigation measures all create new breeding grounds for mosquitoes. Crises, environmental disasters and wars usher in a host of rodents and arthropods, now precipitously closer to humans. Additionally, population density growth in large cities and metroplexes, rural displacement, and global air traffic and other modes of travel are important factors in establishing such new connections or can affect the ecological cycles regarding these viruses [3]. Viruses, especially RNA viruses with their ability to adapt quickly to changing environmental conditions, are among the most outstanding examples of emerging zoonotic diseases. In consideration of all this, the threat of viral zoonoses is still far from being under control, despite all the advances in modern scientific research and medicine. Our protection is dependent upon our ability to identify emerging zoonotic diseases as soon as possible and develop new vaccines, antiviral therapies and drug resistance. The World Health Organization (WHO) has created a list to prioritize research and development referencing these viruses which are important in the public health emergence context: blueprint R&D list. Presently, the priority diseases are COVID-19, Crimean-Congo hemorrhagic fever virus, Ebola virus disease and Marburg virus disease, Lassa virus, Middle East respiratory syndrome coronavirus (MERS-CoV) and Severe Acute Respiratory Syndrome (SARS), Nipah and henipaviral diseases, Rift Valley fever, Zika virus and Disease X.

Crimean-Congo hemorrhagic fever virus (CCHFV) is the focus of this dissertation due to its being one of the significant zoonotic agents. Notably, CCHFV is listed as a priority in the WHO R&D blueprint list. CCHFV is classified as an emerging disease by major epidemiological organizations around the world due to the increasing number of cases reported in recent decades [4], [5]. Due to the epidemiological potential of the virus, the high mortality rate is 5-30% regarding the disease [6], the risk of nosocomial infections, and the difficulties in preventing and treating the disease, CCHFV poses a serious challenge to health-epidemiological care systems. Therefore, the WHO has made epidemiological preparedness, research and development regarding CCHFV a priority. Trends in annual and periodic human CCHF cases and mortality rates have gradually increased in recent decades [7]. Thus, CCHFV content research (epidemiology, pathogenesis, molecular characteristics, clinical aspects, diagnosis, antiviral research and vaccine development) has also become increasingly relevant. However, research on CCHFV is hampered by the high infectivity of the virus, which allows the virus to be treated only under the highest biosafety level (BSL-4) [8]. Additionally, laboratory studies on transmission and pathogenicity of CCHFV are further limited by the lack of appropriate animal models [9].

Considering the geographic burden associated with CCHF, it is a widespread tick-borne virus throughout the world. Virus isolation, detection and/or disease have been reported from more than forty countries throughout Europe, Asia, Africa, the Middle East, and India (Figure 1). This geographic range is the most extensive one among the medically important tick-borne viruses.

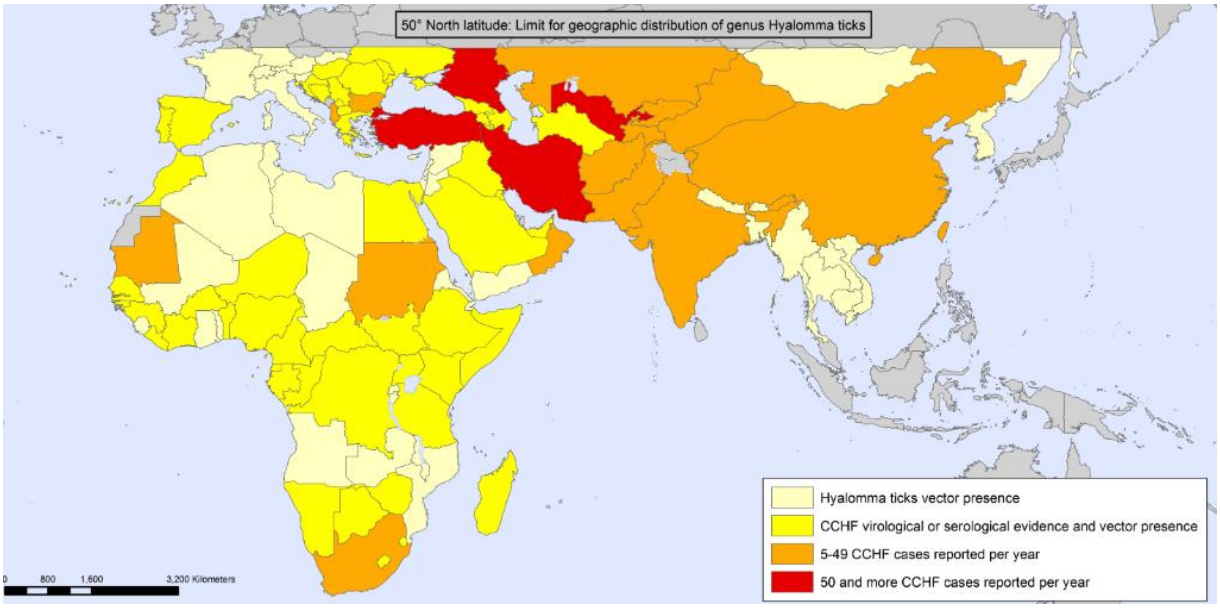

Figure 1: *CCHFV distribution map by WHO, 2017.*

Several species are susceptible to CCHF infection including humans, livestock, small vertebrates, and ostriches. CCHFV is transmitted to humans through a tick bite and/or exposure to the blood of infected livestock or direct contact with body fluids and the blood of the viremic individual.

Currently, there is no antiviral drug or vaccine approved for CCHF treatment and prevention, however, immunotherapy and ribavirin are proven to be effective during sporadic outbreaks of disease. The development of effective treatments for the prevention of CCHFV caused disease is now a priority for both public health and biodefense agencies.

1.1 History of Crimean-Congo hemorrhagic fever virus

The disease caused by CCHFV was first described in Tajikistan, in which a severe hemorrhagic illness was attributed to the bite of a tick. The disease was described as a case of hemorrhagic fever highlighted by the presence of blood in the urine, rectum, gums, vomitus, sputum and abdominal cavity [10]. The arthropod which caused the disease was said to be related to a louse or tick, which normally is a parasite associated with blackbirds. Moreover, similar diseases were known in other parts of Central Asia [11]. The described arthropods may well have been a species of *Hyalomma* tick larvae, however, not until the late 1960s were researchers able to manage and isolate in which ticks are the vectors of CCHFV [12].

The virus itself was identified during the 1944 outbreak by Soviet scientists and involved more than 200 cases of CCHF which afflicted Soviet Military Personnel in Crimea, Russia [10]. They referred to it Crimean hemorrhagic fever (CHF) due to its symptoms. Later in 1969, it was confirmed in which the same virus caused similar bleeding fever diseases throughout the Democratic Republic of Congo, in which the name, CCHF was adopted soon afterward [10]. The common antigenic structure among Eurasian Crimean hemorrhagic fever strains [13], Asian [14], and African strains of Congo virus [15], [16] resulted in defining the virus as the Crimean-Congo hemorrhagic fever virus [10].

Acceptance of CCHF as a discrete human illness by modern clinicians may have originally lagged since cases tend to occur individually and sporadically in rural areas. Furthermore, many patients develop a mild, nonspecific illness, without a recognizable hemorrhagic fever syndrome [17].

Following the description of CHF (after more than two decades), laboratory research was limited by the failure to culture its causative agent. To cultivate the virus, suckling mice were used. In 1967, blood and tissue samples of a patient were used by intracerebral inoculation of newborn mice and CCHFV was isolated [10]. Antigen production for immunologic assays led to a rapid increase in research [18]: human and animal sera were tested for the presence of virus-specific antibodies. Based on seroprevalence studies from different regions of the world [19] the overall seropositivity of animals (mainly in cattle, sheep, goats, cows and camels) is 0.3-85.7% [20]–[24], and 0.1-85 % of human populations (mainly at-risk professionals) studied was seropositive [25], [26], respectively. The extent of seroprevalence data is influenced by the geographical area in which the study was conducted, the animals involved, and the range of activities performed by humans.

1.1.1 Crimean-Congo hemorrhagic fever virus surveillance in Europe

CCHFV is now considered an emerging or re-emerging pathogen in several European countries. The incidence of CCHFV has increased over the last two decades, mainly in the countries of the Eurasian region, in which sporadic cases and major epidemics have occurred. More than 1000 human cases annually are reported from Southeast European countries and Turkey [27]. CCHFV is endemic in the Balkans (Albania, Kosovo and Bulgaria), in which cases have been consistently reported since the 1950s [27]–[29]. Additionally, CCHFV is considered endemic in Russia, in which after almost 27 years in the south-eastern region, a large number of cases have been consistently

reported since 1999 [30]. In the 2000s, the disease appeared in Turkey, Greece, and most recently in 2016, in Spain. In Europe, human cases have been reported in Albania, Bulgaria, Kosovo, Russia, Serbia, Turkey, Ukraine, Greece, Georgia and Spain [30].

Turkey is a special region of the world regarding CCHFV as the virus was not present in this region until the 2000s, however, since the first detection of CCHFV in 2002, it has been one of the most important CCHVF hotspots on the globe [29]. Extensive studies have been carried out regarding the spread of the virus in the regions of the country which were most affected by the CCHF disease. During these studies in the central and eastern hyperendemic regions of the country, 80% seropositivity was observed in domestic animals [31] and 10% among humans [32]. However, the studies conducted in Turkey demonstrated high variability. Seroprevalence in most studies in Turkey was 19.6% in endemic areas [33].

The presence of CCHFV was first confirmed in Greece in 1975, isolating the virus strain from an individual of the tick species, *Rhipicephalus bursa,* collected from domestic animals [29]. However, the first human case was reported only in 2008, despite a high seroprevalence (4.2% on average) as a result of a nationwide serological survey, which reached 27.5% in some regions of the country [26]. Greece has a high seroprevalence, though only one death associated with CCHFV has been reported [34].

In Spain, the virus was first detected in 2010 from *Hyalomma* ticks which were collected from wild animals [35]. CCHFV was detected in 2.78% of ticks which were collected from wild animals, domestic animals and vegetation between 2011 and 2015. Therefore, the risk of infection in Spain is considered low. Negative seroepidemiological results were also found in Spain from serum samples of humans [35].

CCHFV is a genetically diverse virus among arboviruses. CCHFV nucleotide sequences frequently differ between strains [6]. Ranging from a 20% variability at the nucleotide level between different strains S segment, 31% for the M segment, and 22% divergence between the L segment [36]. Currently, CCHFV strains are classified into seven genetic lineages of which three genetic lineages are present throughout Europe. Lineage Europe 1, which contains pathogenic CCHFV strains, lineage Europe 2 consisting of AP92 and AP92-like strains (e.g., Turkey), and Africa 3 strains (e.g., Spain). Lineage Europe 1 strain has already been found in Albania, Bulgaria, Kosovo, Russia and Turkey [21], [31], [37]–[39]. Lineage Europe 2 has already been found in Greece, Turkey, Kosovo and Algeria [40]–[43]. However, there are countries (Bulgaria, Turkey and Kosovo) in which both lineages can be found [38].

Phylogenetic studies suggest current virus strains evolved from a common ancestor which existed nearly 3000 years ago [36]. The root of the phylogenetic tree can be traced back to Central or West Africa [44]. The virus spread to Central and South Asia in the Middle Ages, in which it reached the Balkan region and Turkey several hundred years ago. As a result, Europe 1 strains were able to spread in these regions [44]. Lineage Europe 2 is a completely genetically distinct group, according to phylogenetic analyzes performed for all three segments, in which it was separated from the other groups close to the root of the phylogenetic tree. The CCHFV AP92 strain was isolated from *Rhipicephalus bursa* ticks collected from goats in Northern Greece [45]. The AP92 strain exhibited a high genetic difference when compared with all known CCHFV strains. Due to this, AP92 has formed a separate group named lineage Europe 2. The circulation of this specific CCHFV lineage in Greece, especially in a region in which the seroprevalence is high, together with the lack of human CCHF cases, implies a probable antigenic, yet non- or low-pathogenic character of this lineage. However, a fatal case due to AP92-like strain has been recently reported in Iran [46].

1.1.2 Crimean-Congo hemorrhagic fever virus surveillance in Hungary

The presence of CCHFV was indicated in Hungary in the last several decades [20], [47]. To the best of our knowledge, the occurrence of CCHFV in Hungary has limited these historical observations [20], [47]. Serological studies conducted in the late 1960s identified sheep, cattle and human samples sera positive for CCHFV in 31%, 0.9%, and 2.9%, respectively [20]. As a part of a national survey for endemic foci of arboviruses, two strains of the virus were isolated from *Ixodes ricinus* ticks in suckling mice during the early 1970s in Hungary [47]. In 2011, twelve positive brown hare serum samples were found out of 198 samples, and (6%) tested parallel with ELISA and IFAT [48]. Since then, no systematic examination has been conducted regarding CCHFV, raising the question of whether these viruses still circulate throughout Hungary. Furthermore, the highly lethal nature associated with CCHFV has restricted research to biosafety level-4 (BSL-4) laboratories and consequently, limited additional research investigation.

Hyalomma tick species, which are the main vectors of the virus, have been discovered in Hungary, thus far. In 2012, adult specimens of *Hyalomma marginatum* were collected from cattle [49], [50]. Moreover, *Rhipicephalus* and *Dermacentor* tick species, which are thought to be additional vectors of the virus, are permanently present in the

country. *Ixodes ricinus* is one of the most common tick species in Hungary [51], however, it remains questionable whether it plays a role as a vector in CCHFV transmission [48].

The perception indicates a need for large-scale active surveillance to effectively estimate the potential public health risk regarding CCHFV in Hungary. Moreover, genetic detection of the virus ideally provides information referencing the strains circulating throughout the country. Hungary lies along the northern boundary line of the spread of CCHFV based on WHO's data. Although there have been no molecularly proven cases of CCHFV infection among humans thus far, the possibility of the debilitating illness may occur in this non-endemic region based on previous serological investigations.

1.2 The molecular characteristics of Crimean-Congo hemorrhagic fever virus

Crimean-Congo hemorrhagic fever virus (CCHFV) is categorically associated with the *Orthonairovirus* genus, the *Nairoviridae* family in the *Bunyavirales* order. CCHFV is a human pathogen and may cause severe hemorrhagic fever with a high fatality rate.

CCHFV is characterized by a tripartite single-stranded RNA genome (S, M and L segment) of ambisense (S) and negative (M, L) polarity. Non-coding regions (NCRs) are present at the 5’ and 3’ termini which are necessary for the viral RNA-dependent RNA polymerase to bind and initiate transcription and/or replication of the viral genome. The three genome segments encode four structural proteins: the RNA dependent RNA polymerase is encoded by the large (L) segment, the glycoproteins (Gn and Gc) are encoded by the medium (M) segment, and the nucleocapsid protein (NP) and nonstructural protein (NSs) are encoded by the small (S) segment [52]. The genome segments with NP and L protein are encapsidated in genomic ribonucleoprotein complexes (RNP) which are packaged into viral particles. Lipid envelopes and surface glycoproteins (Gn, Gc) are organized in these viral particles.

CCHFV assembly has not yet been clarified. CCHFV RNPs are mostly found in the cytoplasm, while NP is localized in the perinuclear region which is close to the Golgi complex. Glycoproteins evolve through post-translational modifications in both Golgi-complex and trans-Golgi-networks (TGN). The assembly of these particles occurs in Golgi-complex and TGN.

The cellular receptor of CCHFV which is required for cell entry is currently unknown. However, the *Hantavirus* genus (which also belongs to *Bunyavirales* order) binds to host cell integrins before being internalized by clathrin-dependent endocytosis [53]. CCHFV also is endocytosed through a clathrin-mediated endocytosis mechanism.

During entry, clathrin and the clathrin pit adaptor protein-2 complex are needed, however, not caveolin-1 [54]. Moreover, CCHFV entry and replication are also dependent on cholesterol and low pH [55]. Following cell entry, virus particles are transported to early endosomes and then onto multivesicular bodies (MVB).

1.3 Current status of Crimean-Congo hemorrhagic fever prevention, risk reduction and addressing the role of ticks

Based on epidemiological data thus far, there are dedicated professions considered as high risk regarding CCHF infection: health care workers, shepherds, farmers, veterinarians, slaughterhouse workers, soldiers, outdoor sports enthusiasts and campers. Another group at risk are relatives and friends of afflicted individuals prior to hospitalization, treatment and isolation. In endemic areas, all individuals living in a rural environment or travelling from cities to the countryside are at risk of infection. Contact with viremic animals or human patients acts as another risk factor. To manage CCHF disease, individuals who are in high-risk groups must be educated regarding routes of transmission and prevention of CCHF [56].

The survival and replication of CCHFV occurs in nature in the tick-vertebrate-tick enzootic cycle (Figure 2). During the life cycle of hard ticks belonging to the *Ixodidae* family, their transformation between their three developmental stages (larvae, nymphs and adults), and the laying of eggs requires the blood of vertebrate hosts, from which they can feed once or over several days or even over several weeks at each stage of their development [35]. Vector tick species pick up the virus in any of their life cycles when feeding on a viremic host or when co-feeding with an infected tick on a non-viremic host. Ticks feed on a wide variety of vertebrate hosts, with specific tick-host cycles strongly influencing CCHFV circulation in endemic areas [6]. The larvae and nymphs of *Hyalomma* ticks feed on small vertebrates, mostly rodents, rabbits and terrestrial birds, while adults feed on large mammals, primarily ungulates [6]. In mammals, followed by rapid viremia the presence of anti-CCHFV antibodies is detectable [57]. During the viremic period, the virus can be transmitted to other uninfected ticks, forming a bridge for the virus to survive between ticks [58]. Additionally, in the case of ticks, transovarial and transstadial transmission have been shown, including transmission during copulation with less efficiency [57]. The presence of a stable population of the vector tick species in the region is an essential condition for the emergence of the virus in a new area [58]. One of the main determinants of this is the basic climate of a given region [59].

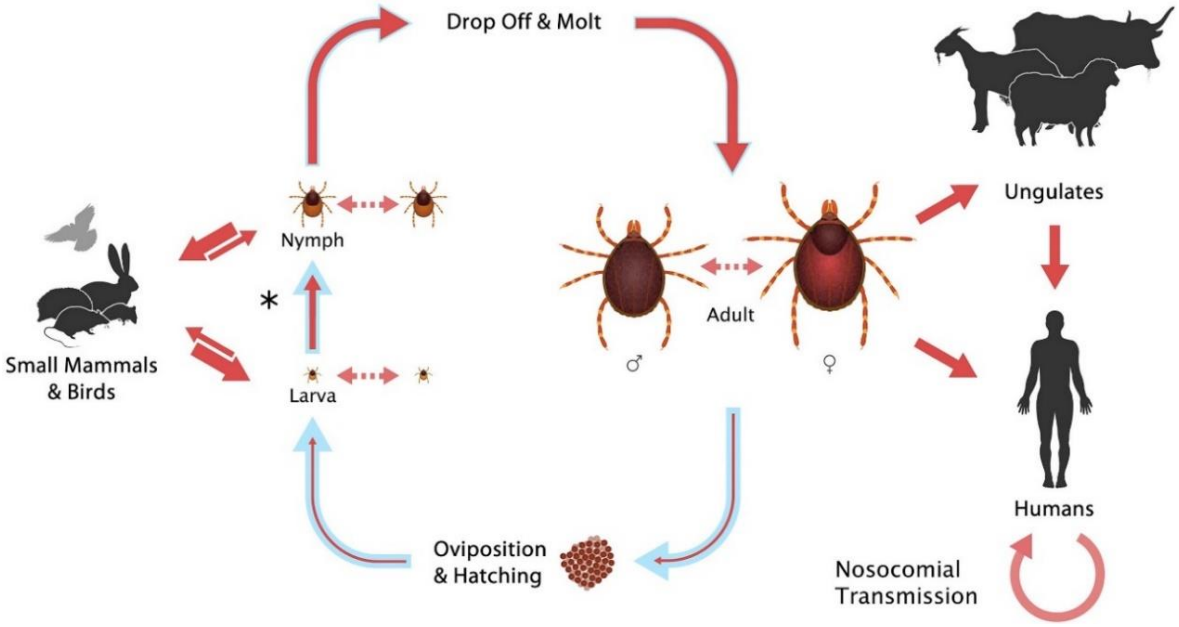

Figure 2: *Overview of the tick-vertebrate-tick enzootic cycle [58]. Blue arrows indicate the tick's life cycle. Red arrows mark possible transmission of CCHFV between ticks and mammals, or transmission between co-feeding ticks.*

CCHFV has been detected in a wide range of tick species thus far. More than thirty species of ticks have been identified as potential vectors, representing several tick genera, namely, the *Hyalomma, Rhipicephalus, Ornithodoros, Dermacentor* and *Ixodes*. Although the virus has been associated with a wide variety of tick species, it is believed members of the *Hyalomma* genus are mainly involved in transmitting the causative agent [11], [35], [60]. To control CCHF disease, monitoring programs in ticks and animals should be executed to define endemic or high-risk areas and to detect virus spread at an early stage [61].

From the early 2000s, Turkey revealed how areas with higher levels of CCHF cases were significantly associated with zones of high climate suitability for *Hyalomma* ticks and a high rate of fragmentation of agricultural land [62]. CCHFV was detected in 20% of *Hyalomma* ticks collected from the region [62]. Additionally, the presence of CCHFV has been detected in different tick species (*Rhipicephalus bursa* and *Dermacentor marginatus*) in many European countries (Greece [29], Spain [35], Turkey [63] and Albania [6]).

Ecological changes and displacement of human and animal populations are the major drivers regarding the distribution of CCHFV. Moreover, changes in the environment also help the spread of CCHFV. Climate and anthropogenic factors (changes in land use, deforestation, agriculture and hunting activities) may also help the spread of infectious diseases [64], especially if these changes affect the vector as well. As a result of climate change, rising temperatures and a decrease in precipitation in the Mediterranean region, according to a prediction of a previously published ecological model, will result in a sudden increase of the population of *Hyalomma marginatum* in suitable habitats and the spread north (Figure 3) [65]. The geographical distribution of CCHFV in Eurasia strongly correlates with *Hyalomma* species, furthermore, this tick species can be continuously associated with existing human diseases and are of key importance in maintaining endemic areas of the virus. Additionally, the spread of Mediterranean tick species is also assisted by the movement of migratory birds through disseminating the ticks under favorable climatic conditions [66]. To protect the public from CCHF disease is dependent upon individual prevention. Humans should cover their bodies with clothing and when possible, avoid visiting tick habitats during the active tick season. Moreover, tick repellents should be applied. Education of the general public regarding the mode of transmission through tick bites, handling the ticks, caring and butchering of animals and the means for personal protection is deemed absolutely necessary.

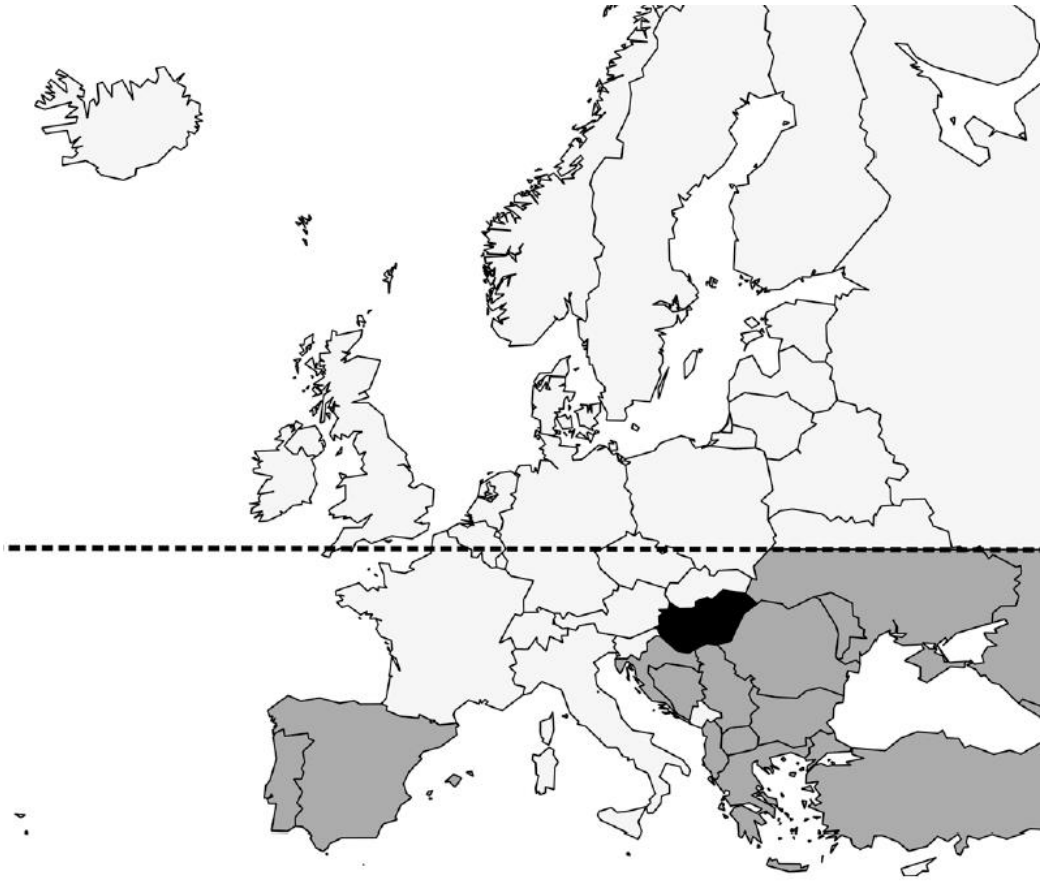

Figure 3: *Climatic and geographical distribution limits of Hyalomma ticks are depicted with a dashed line [67]. Grey countries in Europe have virological or serological evidence and vector presence based on the WHO's data. Hungary is indicated in black.*

1.4. Potential antiviral therapy combating Crimean-Congo hemorrhagic fever virus: RNA interference

Among the RNA-based therapies, RNA interference (RNAi) appears promising against many viruses *in vitro* studies [68]–[70]. In the case of CCHFV a novel, effective therapy is needed. Currently, there is no effective vaccine against CCHFV. Ribavirin and favipiravir have promising results, both *in vitro* and *in vivo* [71], [72]. The efficacy of other options including Intravenous Immunoglobulin (IVIG), steroids, CCHF hyperimmuneglobulin, and CCHF monoclonal antibodies are still controversial [73]. The novelty of this thesis also originates from the fact RNAi has not yet been applied in the case of CCHFV. Since CCHFV is highly pathogenic and must be handled in a BSL-4 laboratory, very few laboratories are involved in CCHFV research and the inhibitory effect

of siRNAs has not been used against it in any other studies. This research complies with WHO research and development goals of priority diseases.

The RNA interference pathway was originally recognized in *Caenorhabditis elegans* as a response to dsRNA presence leading to sequence-specific mRNA cleavage [74]. It soon turned out RNA interference is not restricted to nematodes and can be induced in various other taxa, such as *Drosophila melanogaster*, *Trypanosoma*, and vertebrates [75]. This discovery had been preceded by the observation of similar phenomena in plants and fungi although the involvement of dsRNA was uncertain at the time. For instance, in petunia, the introduction of exogenous transgenes silenced the expression of the homologous endogenous loci. These phenomena were referred to as, 'co-suppression' (also termed post-transcriptional gene silencing, PTGS) in plants and 'quelling' in fungi. This wide range of silencing pathways is now collectively known as 'RNA silencing'. Although the term 'RNA interference' is also often used to indicate 'small RNA-mediate silencing phenomena' in general, it usually means only the mRNA cleavage event induced by the administration of dsRNA. RNA interference can also refer to the technology in which small RNA is used as an experimental tool to shut off gene expression [75].

Small RNAs, either exogenous small interfering RNAs (siRNAs) or endogenous microRNAs (miRNAs), are taken up by a cytoplasmic RNA-induced silencing complex (RISC), which cleaves one strand, leaving the remaining unpaired guide strand to search for mRNAs bearing complementary sequences. Once recognized, if the target site on the mRNA has nearly perfect complementarity to the guide siRNA, the mRNA is cut by an Argonaute endonuclease in the RISC and then degraded, silencing the expression of the protein it encodes. Typically, protein expression is reduced yet not completely eliminated (Figure 4). The RNA interference machinery is present in all cells, where it is used to regulate the expression of key genes involved in cell development, differentiation, and survival. Small RNAs can be readily designed to target any gene, whether an endogenous host gene or a foreign viral gene [76].

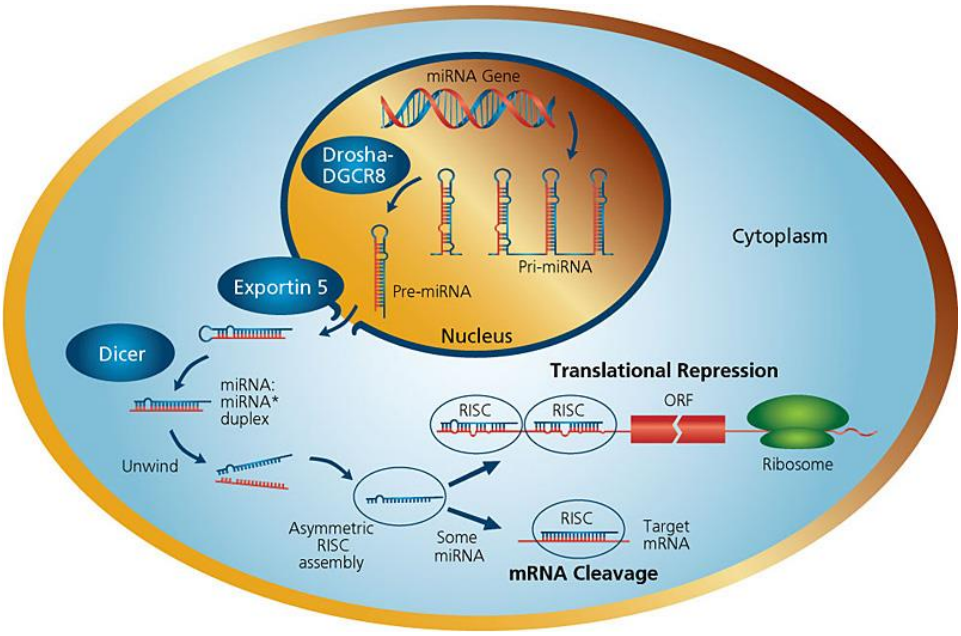

Figure 4: *Mechanism of RNA interference [77].*

In consideration of antiviral therapies, RNAi can be used to suppress viral replication by targeting either viral- or host genes needed for viral replication. Silencing viral genes such as viral polymerases, master regulators of viral gene transcription, and viral genes which act early in the viral life cycle may suppress viral replication more effectively than targeting late or accessory viral genes. Moreover, RNAi can target viral proteins and pathways, which are unique to the viral life cycle and it has become possible to interfere with viral infections and replication without unacceptable host cell toxicity [76].

Maffioli and colleagues used the Langat virus in the organotypic brain culture model to demonstrate the effectiveness of siRNA in inhibiting the TBE virus [78]. A total of nineteen siRNA sequences targeting genes within the structural (S), the non-structural (NS), and the 3′ and 5′ untranslated regions (UTRs) of the Langat genome were tested. In this study, the majority (11/19) of the nineteen siRNAs were capable of reducing Langat virus titer by more than 80% in comparison to nonsense siRNA when transfected into the cells prior to infection. The most effective reduction in viral replication was achieved with siRNA sequences targeting the 3′ UTR and the structural genes.

Gitlin and colleagues attenuated the infection of poliovirus following transfection with siRNAs which targeted either a capsid-protein mRNA or the viral polymerase mRNA. They also showed mutation of the viral genome within the sequence targeted by siRNAs which led to the emergence of a resistant variant [68].

The West Nile virus is an emerging human pathogen for which specific antiviral therapy has not yet been developed. Geiss and colleagues examined the ability of siRNAs to block the replication of the West Nile virus in human Huh 7.5 cells. A 21 nucleotide region of the WNV capsid gene was targeted. Using the conventional lipid-based delivery system which targets siRNA to the cytoplasm, we confirmed the pretreatment of cells prevented infection. However, resistance to RNA interference was observed when siRNA was delivered following viral translation and replication had commenced. In contrast, when siRNA was delivered by electroporation, a technique that allows macromolecules to pass across intracellular membranes, it reduced viral replication in a sequence-specific manner even if active replication was already underway. Hence, they found that the cellular localization of siRNA appears more important than the absolute amount of siRNA delivered into the cell towards determining its effectiveness against actively replicating WNV RNA [79].

Another study used chemically synthesized siRNAs to target the mRNA of the Hazara virus produced by the three genomic segments [68]. They demonstrated how the siRNAs targeting the NP mRNA displayed a stronger antiviral effect than those complementary to the L and M transcripts in A549 cells. SiRNAs were efficient when transfected in cells before or after HAZV infection and their use in combination with ribavirin induced a synergistic or an additive antiviral effect, according to the dose of ribavirin used. Hazara virus (HAZV) belongs to the Crimean-Congo hemorrhagic fever (CCHF) serogroup which also includes CCHF virus (CCHFV), a major pathogen regarding humans. HAZV is a superb model to study CCHFV due to a close serological and phylogenetical relationship and a classification allowing handling in a BSL-2 laboratory.

RNA interference has several advantages. As with all RNA-based technologies, the response time to therapeutic options is rapid. If the RNA viral genome changes, the solution to adaptation is easier and most importantly, the manufacturing process is much simpler than as in the case of a complex chemical.

In the case of CCHFV, there are neither vaccines nor effective antiviral therapies for the treatment of infections in humans [80]. Accordingly, the risk of viral infection will presumably increase in the forthcoming years, research projects aimed at prevention and treatment in treating a possible infection will be vital. Viral resistance, sequence diversity, and drug toxicity pose significant challenges regarding for all antiviral therapies. In the case of CCHFV, it must be handled in a BSL-4 laboratory due to its high pathogenicity.

2. AIMS OF THE STUDY

The first goal of this thesis was the evaluation of the risk of emergence in Hungary of the CCHF virus by the evaluation of CCHF seroprevalence in nature among wild rodents.

- Producing CCHFV recombinant nucleocapsid protein using *Escherichia coli* expression system.
- C57BL/6 mice immunization with CCHFV recombinant nucleoprotein for polyclonal antibody production.
- Optimization of immunoassays (dot-blot, Western-blot and immunofluorescence assay) using the newly produced CCHFV recombinant nucleocapsid protein and polyclonal antibodies.
- Dot-blot pre-screening and immunofluorescence confirmation of rodent serum samples for CCHFV.
- CCHFV seroprevalence study in local rodent populations.

The second goal of the work was the development of CCHF treatment, based on the siRNA antiviral technology:

- Design and synthesis of highly effective siRNAs with the Whitehead siRNA Selection Program based on sequences of CCHFV Kosova Hoti strain S, M, and L genomic segments.
- siRNA delivery into cells with high transfection efficiency and optimization of measurable transfection controls.
- Discover the appropriate concentration of siRNAs which effectively inhibits CCHFV replication and does not cause a cytotoxic effect in cells.
- Optimization of RT-ddPCR system for CCHFV with an ideally measurable siRNA inhibitory effect.
- Determine which siRNAs have a significant inhibitory effect regarding CCHFV replication.

3. MATERIALS AND METHODS

3.1 Rodent samples collection for serological tests

The forest reserve of Kőszegi-forrás (46°09′28.88″ N, 18°17′09.90″E) is located in the Mecsek Middle Mountain Range in Southwest Hungary and includes a total area of 149 ha (Figure 5). The forest reserve is located upon a plateau surrounded by a deep valley, with an elevation of 320–400m above sea level. No fewer than 13 sampling plots were selected in 2011, 2012, and 2013 in an area roughly measuring 11 ha. The sampling was part of a previous ecological study and serological survey [81], therefore sampling points were set up based on the former investigation. A capture–mark–recapture method (CMR) was used, implementing the use of live trapping plastic box traps. Various forms of cereals and bacon mixed with aniseed extract and vegetable oil were used in the form of bait. Each sampling plot included a grid comprised of 6×6 trap stations, 5m apart. The monitoring of small mammals was performed in seven monthly trapping periods during eight months, spanning from March through October, in each of the corresponding years. In each month, standard five-night capture events were carried out. The traps were examined twice a day, first at 7 AM, then again at 7 PM. Blood samples were taken from trapped animals each month using retro-orbital sampling. In each month during the five-night capture period, a blood sample from a given individual animal was taken once.

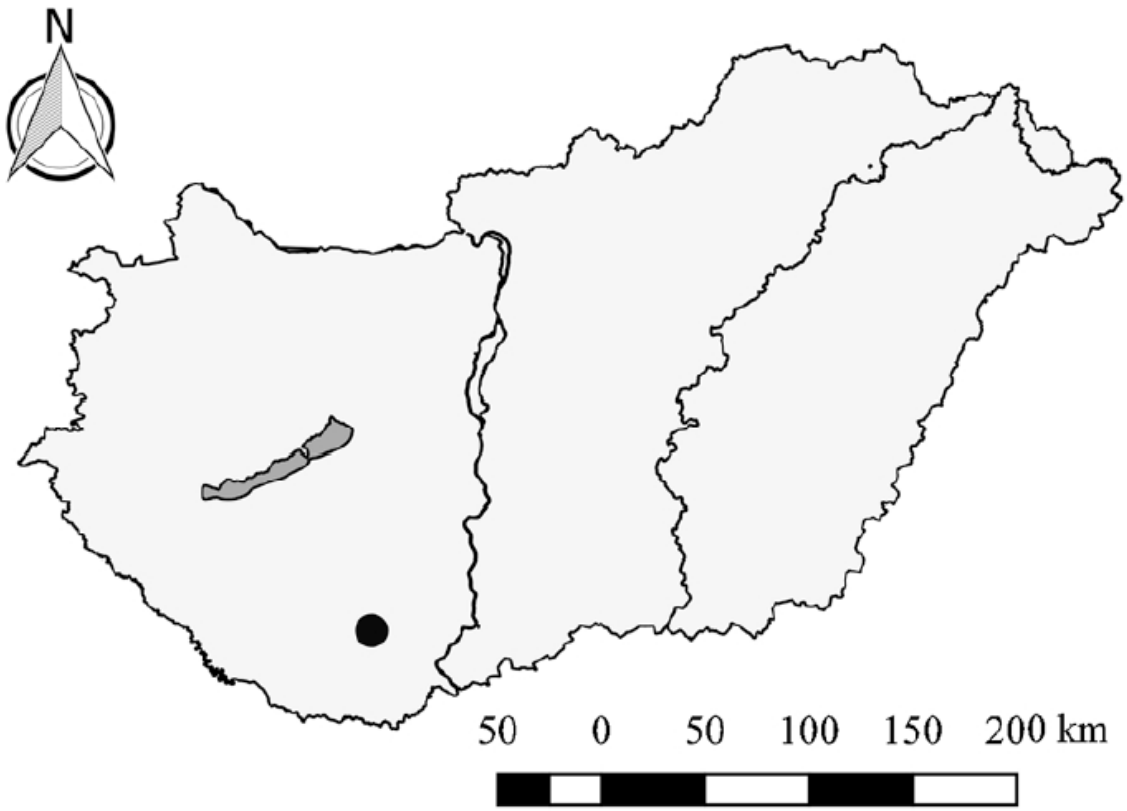

Figure 5: *Location of the study area in Hungary [67]. The Kőszegi-forrás forest reserve is annotated in the use of a solid black dot.*

3.2 Production of the recombinant CCHFV nucleoprotein

3.2.1 Existing CCHFV construct

The 1448-bp-long nucleocapsid protein fragment of the IbAr10200 Nigerian strain of CCHFV was produced with an *Escherichia coli* protein expression system (Figure 6).

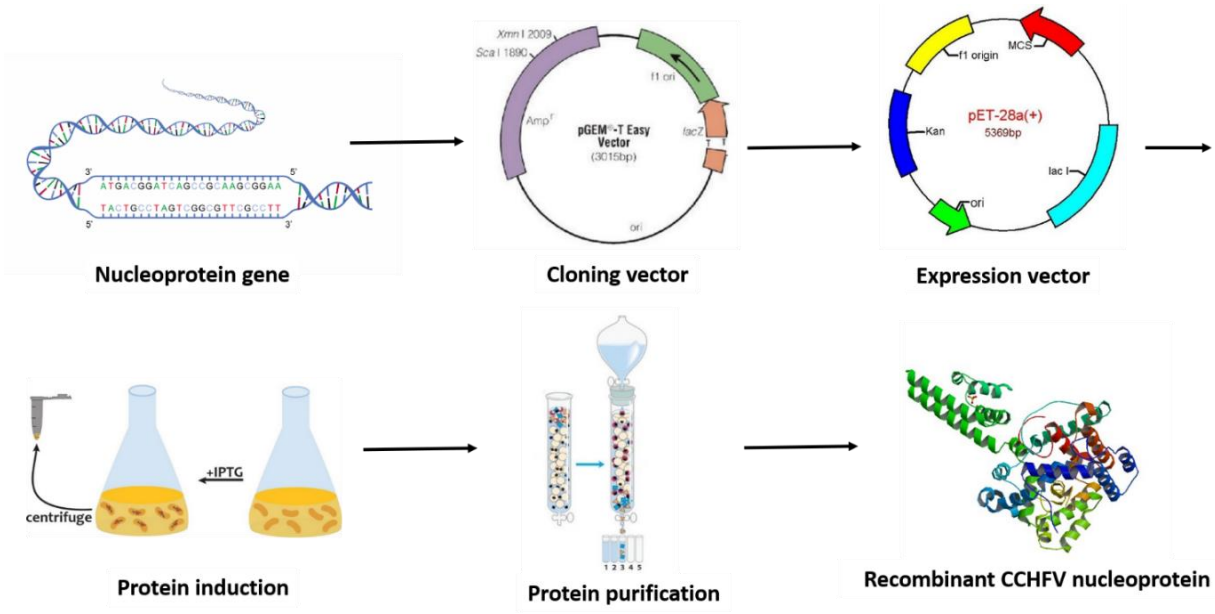

Figure 6: *Flow chart of recombinant CCHFV nucleoprotein production.*

BL21 Rosetta (DE3) competent cells (Novagen) were used which contain the recombinant pET28a plasmid (Novagen) with nucleocapsid sequence. The construct "pET28a(+)-CCHFV" transformed into *E. coli* was produced previously as described by our group [82]. I worked with this finished construction and protocol to produce recombinant nucleocapsid protein during my PhD work.

3.2.2 CCHFV nucleocapsid protein expression

Competent cells containing the recombinant pET28a plasmid were cultured in a LB medium containing 34 µg/ml chloramphenicol and 30 µg/ml kanamycin in a rotary shaker 200 rpm at 37° C. Once cells reached an OD_{600} of 0.8-1 log phase, they were induced with 1 mM IPTG and incubated at 37° C for three hours. The cell pellet was collected by centrifugation at 4000 g for twenty minutes.

3.2.3 CCHFV nucleocapsid protein purification and refolding

Bacterial Protein Extraction Reagent® (B-PER, Thermo Scientific) was used to resuspend induced bacterial cells in full accordance to the manufacturers' recommendations.

The recombinant protein was purified under denaturing conditions, utilizing HIS Select HF Nickel Affinity Gel column (Sigma Aldrich). CCHFV recombinant nucleoprotein was bound to the affinity gel at 4° C, flowing the lysate three times through the column. Elution was carried out by a descending pH elution agent (8 M urea, 0.1 M NaH_2PO_4, 0.01 m Tris), decreasing pH by 0.5 from pH 8 to pH 4. The flow-through and elution fractions were both separated by SDS-polyacrylamide gel electrophoresis (mini-PROTEAN® TGX, Bio-Rad) and visualized by Coomassie blue staining (Brillant Blue R, Sigma) and Western-blot assay.

Proteins were renatured via dialysis. We used a 12.4 kDa dialysis membrane (Sigma). Urea concentration of the original elution buffer was gradually decreased by applying increasingly diluted dialysis buffer (6 M urea, 20 mM HEPES, pH=7.9; 300 mM KCl, 5 mM $MgCl_2$, 10 % glycerol, 0.5 mM PMSF) during the dialysis process.

The exact recombinant protein size was determined by SDS-polyacrylamide gel electrophoresis (Mini-PROTEAN® TGX, Bio-Rad) and visualized by Coomassie blue staining (Brillant Blue R, Sigma) and Western-blot assay. Protein concentration was determined using Qubit Protein Assay Kit™ (Thermo Fisher) on Qubit Fluorometer according to the manufacturers' instructions.

3.3 Mice immunization for polyclonal antibodies

C57BL/6 wild-type mice were immunized to produce polyclonal antibodies against the recombinant nucleocapsid protein of IbAr10200 Nigerian strain of CCHFV. The purified recombinant CCHFV nucleocapsid protein was diluted in 1×phosphate-buffered saline (PBS) (Lonza) and was mixed with complete Freund adjuvant (Sigma) in a 1:1 ratio. Four sample mice were inoculated intraperitoneally using 100 µl mixture for each specimen. A negative control mouse was inoculated with the same mixture without the recombinant protein. Following four weeks, the recombinant CCHFV nucleocapsid protein was diluted in a solution consisting of 1×PBS and was mixed with incomplete Freund adjuvant (Sigma) in a 1:1 ratio. Four mice were inoculated again, intraperitoneally, with 100 µl mixture per specimen. A negative control mouse was inoculated with the same

mixture without the CCHFV protein. Following two weeks, the tail vein blood collection was performed in support of testing the CCHFV-specific antibody response for Western-blot. Following one week, mice with a positive immune response were given a booster inoculation. 1×PBS and recombinant peptide 2:1 ratio was inoculated intraperitoneally consisting of 100 µl mixture per specimen. Seven days later, the total blood volume was collected from mice through the use of cardiac puncture (Figure 7). Blood was centrifuged (10,000 × g for 10 min) to separate the serum from the cells. Immunized mice serum samples were used as positive controls in future experiments. The immunization protocol was executed following personal communication with Dr. Péter Engelmann (Department of Immunology and Biotechnology, University of Pécs, Pécs, Hungary).

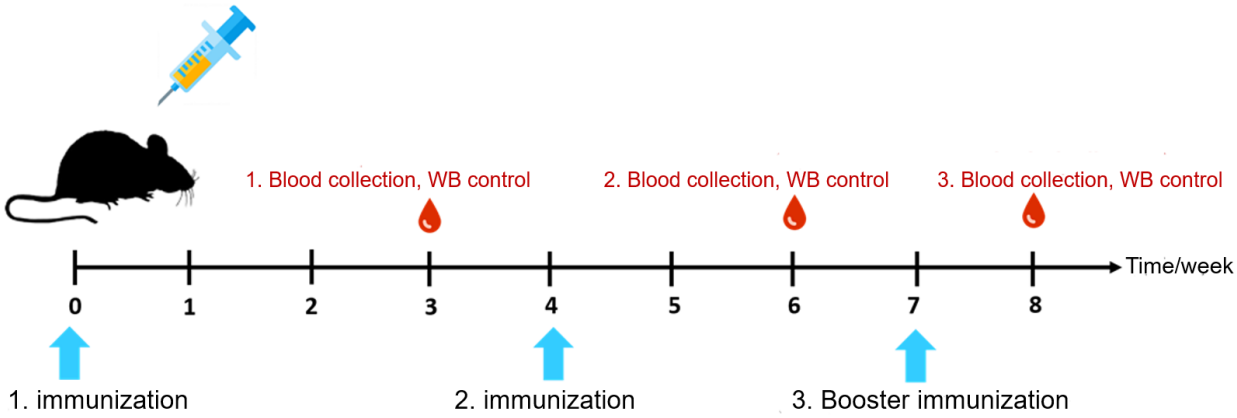

Figure 7: *Timeline and activities of mice immunization.*

3.4 Immunoassays

3.4.1 Western-blot assay

We used Western-blot assay to confirm the quality of CCHFV recombinant nucleoprotein following protein expression and purification and later to confirm CCHFV specific immune response during mice immunization.

The CCHFV recombinant nucleoprotein was loaded into the wells of Mini-PROTEAN® Precast Gels (Bio-Rad). Polyacrylamide gel electrophoresis was carried out at 180V for thirty mins. Following electrophoresis, proteins were transferred to 0.45 µm pore size nitrocellulose membranes (Bio-Rad), using Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad), at 180 mA for thirty minutes. Following a sixty minutes blocking step with 5% non-fat dry milk (Blotting-Grade Blocker, Bio-Rad), immunized mice sera

were applied 1:100 dilution using tris-buffered saline (TBS) pH=7.5, containing 0.1% bovine serum albumin (BSA) and 0.05% Tween-20 (Sigma). Horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG antibody (Dako) was used as a secondary antibody in a 1:800 dilution. All incubation steps were executed at room temperature for one hour. Washing steps (three consecutive ten minutes) were implemented using 1×TBS-T (pH=7.5). The color development was achieved using a 3.3′-diaminobenzidine color development solution (Bio-Rad) based on the manufacturer's instructions. Enhanced chemiluminescence (ECL) staining Western-blotting luminol reagent (Santa Cruz Biotechnology) was also used during Western-blot assays in full accordance to the manufacturer's recommended.

3.4.2 Dot-blot assay

Wild rodent samples were pre-screened using the standard 96 well Bio-Dot® (Bio-Rad) apparatus, based on the manufacturer's instructions regarding the presence of anti−CCHFV immunoglobulin G (IgG) antibodies. In reference to the recombinant CCHFV protein binding, 100 ng/well recombinant protein was used. Rodent samples and positive and negative control mouse samples were used in a 1:100 dilution. Horseradish peroxidase-conjugated rabbit anti-mouse IgG antibody (Dako) was used as a secondary antibody in a 1:1000 dilution. The color development was achieved using a 3.3′-diaminobenzidine color development solution (Bio-Rad) based on the manufacturer's instructions. In regards to the positive control, an immunized mouse serum sample was used as the primary antibody. The negative control was performed using the serum from an uninfected mouse.

3.4.3 Immunofluorescence assay

To effectively confirm the dot-blot positive rodent samples, CCHFV-specific IgG antibodies were detected from rodent serum samples using immunofluorescence assay (IFA). All laboratory manipulations associated with CCHFV were performed in a BSL-4 suite laboratory, aligned to the University of Pécs. 96-well plates were seeded using A549 cells (25,000 cell/well) and infected with a known CCHFV Kosova Hoti strain [83]. Three days following infection, cells were fixed for twenty minutes with ice-cold absolute methanol. Following air-drying and twenty minutes of UV radiation, the plates were removed from the BSL-4 laboratory and placed in storage at 4° C until further use. The plates were equilibrated to room temperature and 1×PBS was added to wash cells. 50 µl

1% BSA diluted in 1×PBS was next added to block false-positive reactions. The plates were incubated for thirty minutes at room temperature. Following the blocking step 50 µl, 1:50 diluted rodent serum samples were added, and the plates were again incubated at 37° C for thirty minutes. Following primary antibody binding, 100 µl 1×PBS was used for five minutes in three instances, to effectively rinse the cells. 50 µl goat anti-mouse IgG Alexa Fluor® 488 (Abcam) was used as a secondary antibody in 1:1000 dilution, followed by three rinse cycles in 100 µl 1×PBS for 5 min. Plates were evaluated using a Nikon Eclipse Ti-U microscope system, at an excitation wavelength of 480 nm. In regards to the positive control, an immunized mouse serum sample was used as a primary antibody. Serum from an uninfected mouse was used as a negative control. In consideration of negative cell control, uninfected cells were used with immunized mouse serum as the primary antibody.

3.5 RNA interference experiments

3.5.1 In vitro virus amplification and titer determination

During the preliminary experiments, the CCHFV Kosova Hoti strain [83] had to be adapted to the cell lines maintained in our laboratory to find the proper one suitable for our experiments. VeroE6, 293A, and A549 cell lines were tested in the BSL-4 laboratory. CCHFV has different cytopathogenic effects on cell lines.

Based on previous *in vitro* propagation tests, we used A549 cells (human lung carcinoma cell line ATCC CCL-185), which were grown in Dulbecco's modified eagle medium (DMEM) (Lonza) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (EuroClone) and 1% Penicillin-Streptomycin (Lonza) maintained at 37° C in a humidified atmosphere containing 5% CO_2.

A549 cells with 60% confluence were infected by the CCHFV Kosova Hoti strain in our experiments. The virus was grown to high titers on A549 cells and the supernatants were aliquoted and were frozen at -80°C in 1 ml vials and constituted the viral stock. All laboratory manipulations associated with infectious CCHFV were performed in a BSL-4 suite laboratory, aligned to the University of Pécs, Szentágothai Research Center.

CCHFV viral stock was titrated using the TCID50 method with the immunofluorescence assay. Briefly, serial 10-fold dilutions of CCHFV supernatant were inoculated (100 µl) on 60% confluent A549 cells (30,000 cells/well) in 48-well plates. Viral adsorption was allowed for one hour at 37° C. After washing cells with PBS three times, cells were incubated for three days at 37° C in DMEM supplemented with 2% FBS. The fixation and the immunofluorescence assay were performed as previously described

using with polyclonal mouse antibody which was produced against the recombinant CCHFV capsid protein [67]. The percentage of infected cells was observed with immunofluorescence microscopy and recorded for each virus dilution, and then results were used to mathematically calculate a TCID50 result with the Spearman-Karber method. During our experiments, A549 cells were infected with CCHFV at a MOI of 0.1 in our following infection and transfection assays.

3.5.2 Design and synthesis of siRNAs

The sequences of CCHFV Kosova Hoti strain S, M, and L genomic segments (GenBank: DQ133507, EU037902, EU044832) were used to design the siRNAs. Synthetic 21-nucleotide siRNAs with short 3' overhangs (UU) were designed by the Whitehead siRNA Selection Program to possess an antisense strand complementary to the CCHFV [84]. siRNA sequences were chosen according to the algorithm score. For each viral mRNAs, five siRNAs were synthesized by Dhramacon™ (Table 1). Sequences were subjected to a BLAST search against GenBank to minimize off-target effects. All lyophilized siRNAs were reconstituted in full accordance to the manufacturer's instruction, aliquoted in 10 μM stock solutions, and were stored at -20° C until further use. The TOX siRNA (siTOX) (Dharmacon™ RNAi technologies, Lafayette, USA) was used to determine transfection efficacy. ON-TARGET plus non-targeting siRNA pool (Dharmacon™ RNAi technologies, Lafayette, USA) was used as a control siRNA which causes minimal changes in treated cells and reflects a baseline cellular response which can be compared to the levels in cells treated with target-specific siRNAs.

Table 1: *List of designed siRNAs against different segments of CCHFV*

siRNA	Position	Sequence	GC %	Thermodynamic Values
siS1	1651-1673	S 5': GCGGCAACGAUAUCUUUGA UU *mRNA: GT GCGGCAACGATATCTTTGA GA* AS 3': UU CGCCGUUGCUAUAGAAACU	47	-6.8 (-13.7, -6.9)
siS2	26-48	S 5': CCACAGUGUUCUCUUGAGU UU *mRNA: GC CCACAGTGTTCTCTTGAGT GT* AS 3': UU GGUGUCACAAGAGAACUCA	47	-1.4 (-10.3, -8.9)
siS3	466-488	S 5': GUUUCCGUGUCAAUGCAAA UU *mRNA: AG GTTTCCGTGTCAATGCAAA CA* AS 3': UU CAAAGGCACAGUUACGUUU	42	0.3 (-7.7, -8.0)
siS5	1240-1262	S 5': CUGUUGCCAAUCCUGAUGA UU *mRNA: CC CTGTTGCCAATCCTGATGA CG* AS 3': UU GACAACGGUUAGGACUACU	47	0.5 (-8.0, -8.5)
siS6	1388-1410	S 5': CAUGGACAUUGUAGCCUCU UU *mRNA: GA CATGGACATTGTAGCCTCT GA* AS 3': UU GUACCUGUAACAUCGGAGA	47	0.7 (-9.2, -9.9)
siM1	1134-1156	S 5': GGGCUUCCUUUCAAUAGAU UU *mRNA: AA GGGCTTCCTTTCAATAGAT TC* AS 3': UU CCCGAAGGAAAGUUAUCUA	42	-6.2 (-13.2, -7.0)
siM2	520-542	S 5': CCCGUAAGGAGUCUAUUGU UU *mRNA: AT CCCGTAAGGAGTCTATTGT CA* AS 3': UU GGGCAUUCCUCAGAUAACA	47	-5.2 (-11.7, -6.5)
siM5	4463-4485	S 5': GCUCUGGUAUCUCCUGUAA UU *mRNA: TA GCTCTGGTATCTCCTGTAA AG* AS 3': UU CGAGACCAUAGAGGACAUU	47	-3.9 (-11.1, -7.2)
siM6	3173-3195	S 5': GUCCAUACGAAGCUCUUGU UU *mRNA: TT GTCCATACGAAGCTCTTGT GC* AS 3': UU CAGGUAUGCUUCGAGAACA	47	-3.7 (-11.2, -7.5)
siM17	19-41	S 5': CACGUCAGUACGUAAGUGU UU *mRNA: GG CACGTCAGTACGTAAGTGT CA* AS 3': UU GUGCAGUCAUGCAUUCACA	47	-0.7 (-9.5, -8.8)
siL1	5264-5286	S 5': CAGGCCUUGAAGUCUUUAA UU *mRNA: GT CAGGCCTTGAAGTCTTTAA TG* AS 3': UU GUCCGGAACUUCAGAAAUU	42	-6.8 (-11.5, -4.7)
siL3	8442-8464	S 5': GCCUCUUGAUAGGCACAAU UU *mRNA: GG GCCTCTTGATAGGCACAAT GT* AS 3': UU CGGAGAACUAUCCGUGUUA	47	-5.8 (-12.3, -6.5)
siL4	10080-10102	S 5': GCCCUAUUUAGGGACAACU UU *mRNA: AA GCCCTATTTAGGGACAACT TG* AS 3': UU CGGGAUAAAUCCCUGUUGA	47	-5.7 (-13.2, -7.5)
siL8	1126-1148	S 5': GGCAUCAUGUUGUCAACAU UU *mRNA: TT GGCATCATGTTGTCAACAT TC* AS 3': UU CCGUAGUACAACAGUUGUA	42	-4.6 (-11.1, -6.5)
siL33	116-138	S 5': CUGGUCAGUAUGUGACCAA UU *mRNA: TG CTGGTCAGTATGTGACCAA CC* AS 3': UU GACCAGUCAUACACUGGUU	47	-1.2 (-10.3, -9.1)

3.5.3 Transfection efficiency

Although siRNAs cannot pass through membranes of most cells by themselves, mucosal tissues are important exceptions. siRNAs mixed with cationic lipids or even by themselves are efficiently taken up by epithelial cells in the lungs and vagina [85]. A549 cell line seems to be a good choice for our experiments since A549 cells are adenocarcinomic human alveolar basal epithelial cells.

For each experiment, transfection efficiency was monitored by transfecting A549 cells with 200 nM of siTOX (Dharmacon^{TM}) under the same experimental conditions. Cells successfully transfected with siTOX underwent under apoptosis and cell death within 24-48 hours. Following three days of incubation, siTOX transfected cells were trypsinized and manually counted using a hematocytometer (Trypan blue exclusion assay). Transfection efficiency was calculated as the ratio between the numbers of viable siTOX-transfected cells versus non-transfected cells.

3.5.4 Cytotoxicity tests

In some cases, the designed siRNAs could interfere with the tested cells' genes (off-target effect) and cause cell death. During the concentration-dependent transfection, the microscopic observation was performed. A549 cells were transfected with different concentrations (ranging from 0.1 nM to 300 nM) of siRNAs. Cells were observed microscopically following transfection at 24, 48, and 72 hours. During the trypan blue exclusion assay, cell deaths and cell morphological changes have been recorded if the siRNAs targeted S, M, or L segments of CCHFV at high siRNA concentration.

In addition to microscopic observation, cell cytotoxicity was examined using a luminescence cell viability assay kit (Promega – Cell Titer Glo Luminescent assay). This method determines the number of viable cells in culture, based on the quantitation of the ATP present. Cells were transfected with different concentrations of siRNAs (ranging from 0.1 nM to 200 nM). The transfection reagent, Lipofectamine RNAiMax (Thermo Fisher Scientific) cytotoxicity, was also tested. The final volume of Lipofectamine RNAiMax was 1.5 µl/well during the luminescent assay. Following seventy-two hours of transfection, the luminescence measurement was performed. The CC50 (cytotoxic concentration of the extracts to cause death to 50% of cells) was calculated using GraphPadPrism version 8.00 software (Graph Pad Software, San Diego California, USA) for non-linear regression.

The use of cytotoxicity tests was important in deteriming the concentration at which siRNAs do not cause cell death however, their concentration is high enough to inhibit virus replication.

3.5.5 Transfection and infection assay

Transfection and infection experiments were performed on A549 cells in the BSL-4 laboratory. A549 cells were seeded in 96-well plates at a density of 2×10^4 cells/well to achieve 60-70% confluent cell monolayers on the following day in a humidified incubator at 37° C with 5% CO_2.

Cells were transfected in triplicate biological replicates with siRNAs in the following final concentrations: 10 nM and 50 nM. Various siRNA concentrations were complexed with the transfection reagent Lipofectamine RNAiMax transfection reagent (Thermo Fisher Scientific) in full accordance to the manufacturer's instructions. The transfection reagent and siRNAs were diluted in Opti-MEM medium (Gibco). The final volume of Lipofectamine RNAiMax was 1.5 µl/well. The transfection mixture was incubated for twenty minutes at RT to allow the formation of siRNA-lipid complexes and 100 µl of the solution was added slowly dropwise to each well. Mock transfected, non-transfected A549 cells were used as controls for the experiments. ON-TARGET plus non-targeting siRNA pool (Dharmacon™ RNAi technologies, Lafayette, USA) was used as a control siRNA. Mock-transfected cells go through the transfection process without the addition of siRNA while non-transfected cells were not treated at all. Sixteen hours post-transfection, cells were gently washed twice with DMEM. Thereafter, transfected cells were infected with CCHFV at a MOI of 0.1. The inoculum was incubated for one hour to allow the absorption of the virus on transfected cells. Cells were then cultivated in DMEM supplemented with 2% FBS, 1% Penicillin-Streptomycin for forty-eight hours. Non-transfected A549 cells which were infected with CCHFV at a MOI of 0.1 were used as positive cell controls. Cell morphology was monitored and 200 µl cell supernatant was harvested prior to nucleic acid extraction.

Virus replication decrease was assessed by determining the number of genome copies in 200 µl cell supernatant by qRT-PCR and RT-ddPCR.

3.5.6 Viral RNA extraction and polymerase chain reaction methods

Template viral RNA from transfected cells and control cells were extracted from 200 µl culture supernatant using DNA/RNA extraction kit (Geneaid), in full accordance to the manufacturer's protocol. The nucleic acid extraction was performed in the BSL-4 suite laboratory. The RNA elution was achieved in a volume of 50 µl of elution buffer and was stored at -80° C until further use.

To investigate the inhibitory effect of all designed siRNAs in different concentrations (ranging from 10 nM to 50 nM), first a qRT-PCR assay was performed as a pre-screen.

The quantitative real-time TaqMan-based assay was carried out using a One-step RT-PCR kit (Qiagen) in the Light Cycler 2.0 system (Roche). The qRT-PCR reaction mixture consisted of 5 µl of 5x QIAGEN OneStep RT-PCR Buffer, 1 µl of dNTP Mix, 1 µl of QIAGEN OneStep RT-PCR Enzyme Mix, 7.75 µl of RNase-Free Water, 3 µl of $MgCl_2$ solution (25 mM, Promega), 1 µl of sample nucleic acid solution and 0.5-0.5 µl of CCHFV specific primers and 0.25 µl of probe which were based on Atkinson and colleague's publication (Table 2) [86] in a final volume of 20 µl. The reaction profile was as follows: reverse transcriptions at 50° C for thirty minutes, initial denaturation at 95° C fifteen minutes, followed by fifty cycles of amplification at 94° C fifteen seconds, 51° C thirty seconds, and 72° C twenty seconds.

Table 2: *Primers and probe information for the CCHF real-time RT-PCR assay based on Atkinson and colleague's publication.*

Primer/probe	**Sequence (5'-3')**	**Nucleotide position**
CCHF S1	TCTCAAAGAAACACGTGCC	1-19
CCHF S122	CCTTTTTGAACTCTTCAAACC	102-122
CCHF probe	(FAM) ACTCAAGGKAACACTGTGGGCGTAAG (BHQ1)	21-46

Following qRT-PCR prescreening, the siRNAs which inhibited CCHFV replication effectively were measured by RT-ddPCR in three time biological repetitions with different concentrations (ranging from 10 nM to 50 nM). QX200 Droplet Digital PCR system (Bio-Rad, CA, USA) was used to determine CCHFV copy number decrease triggered by siRNAs from supernatants. One-Step RT-ddPCR advanced kit for probes (Bio-Rad, CA, USA) was used in our experiments. The RT-ddPCR reaction mixture consisted of 5 µl of a

ddPCR Supermix, 2 µl reverse transcriptase, 1 µl 300 mM DTT, 900 nM CCHFV specific primers and 250 nM probe, 1 µl of sample nucleic acid solution, and nuclease-free H_2O in a final volume of 22 µl. The final concentrations of CCHFV specific primers and probe [86] were the same as for RT-qPCR assays. The entire reaction mixture was loaded into a disposable plastic cartridge (Bio-Rad, CA, USA) together with 70 µl of droplet generation oil for probes (Bio-Rad, CA, USA) and placed in the QX200 Droplet Generator (Bio-Rad, CA, USA). Following processing, the droplets generated from each sample were transferred to a 96-well PCR plate (Bio-Rad CA, USA) and heat-sealed using a PX1TM PCR Plate Sealer (Bio-Rad, CA, USA). PCR amplification was carried out on a C1000 TouchTM Thermal Cycler with 96-Deep Well Reaction Module (Bio-Rad, CA, USA) using a thermal profile and beginning at reverse transcription: 50° C for one hour and 95° C for ten minutes, followed by forty cycles of 95° C for thirty seconds and 55° C for sixty seconds, one cycle of 98° C for ten minutes, ending at 4° C. Following amplification, the plate was loaded on the QX200 Droplet Reader (Bio-Rad, CA, USA) and the droplets from each well of the plate were read automatically. Positive droplets, containing amplification products, were partitioned from negative droplets by applying a fluorescence amplitude threshold in QuantaSoftTM analysis software (Bio-Rad, CA, USA). The threshold line was set manually at 3780 amplitudes for every sample. Quantification of the target molecule was presented as the number of copies per µl of the PCR mix. All siRNAs in different concentrations were tested in three biological replicates. During the PCR reactions (qPCR and ddPCR) the same target segment was used.

3.6 Statistical analysis

Pearson's chi-squared test was performed using the RStudio software for evaluation of serological results of the tested 2085 rodents [87].

All RNA interference experiments were repeated in three biological replicates. In our siRNA inhibition effect study, we compared the antiviral effect of selected effective siRNAs in different concentrations to the positive control to detect significant variations using the Student's t-test. We compared the siRNAs inhibitory effect to the positive control since using the ON-TARGET plus non-targeting siRNA pool did not change the comparison result either. The measured dataset was statistically analyzed in the R environment [87]. The bar plots were created with ggplot2 R package [88]. During PCR reactions (qPCR and ddPCR), three biological replicates of siRNA inhibited CCHFV samples were used and we did not use technical replicates in the case of these siRNAs

inhibited CCHFV samples, since the three biological replicates include the technical replicate. However, the controls were used in three biological and three technical repeats.

3.7 Ethical statement

The South-Transdanubian Inspectorate of Environment Protection, Nature, and Waters Conservation (Hungary) provided an ethical statement allowing the trapping and marking of the rodents throughout the nature reserve area of Kőszegi-forrás.

Immunized mice were housed following the University of Pécs, Szentágothai Research Center animal house ethical rules, and all experimental protocols were approved by the regional Ethical Committee regarding animal research (study project permission number: BA02/2000-10/2018).

4. RESULTS

4.1 Pre-experiment results

4.1.1 Virus adaptation and titer determination

We found A549 cell line is the most appropriate between cell lines for our experiments with CCHFV Kosova Hoti strain. Following seventy-two hours of CCHFV infection, a microscopically observable cytopathogenic effect was detected on A549 cells. In the case of infected cells, spotted areas of massive cell death were observed in contrast to uninfected cells (Figure 8).

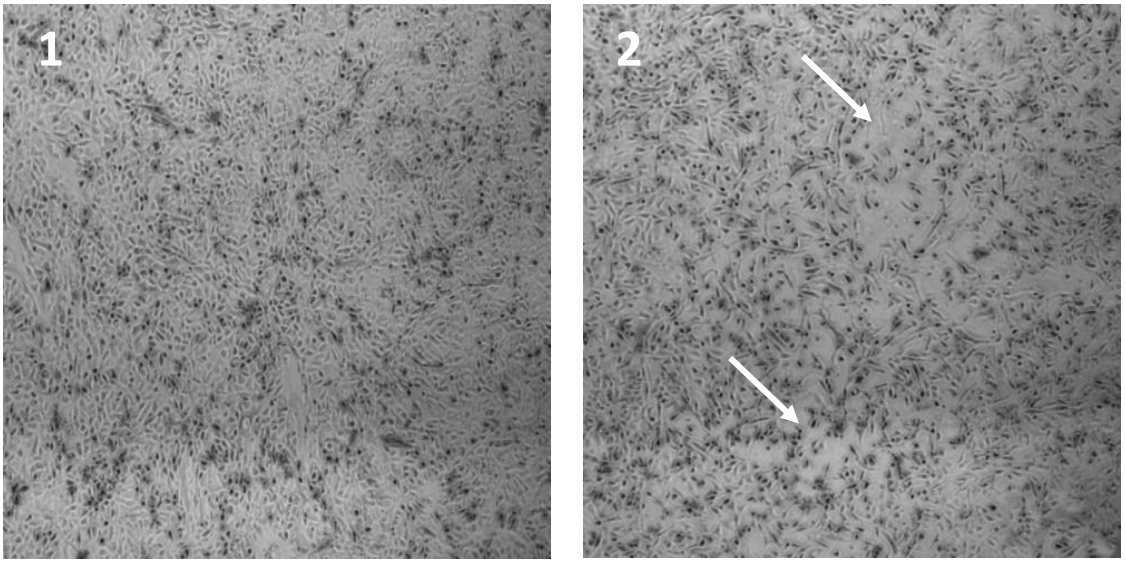

Figure 8: *(1) A549 negative cell control and (2) CCHFV Kosova Hoti strain infected cells following seventy-two hours infection. Cell death is observed in the areas marked with white arrows (100x zoom).*

In addition to microscopic observation, quantitative real-time TaqMan-based PCR was performed to check for virus replication (Figure 9). CCHFV specific primers and probe were used which were based on Atkinson and colleague's publication [86].

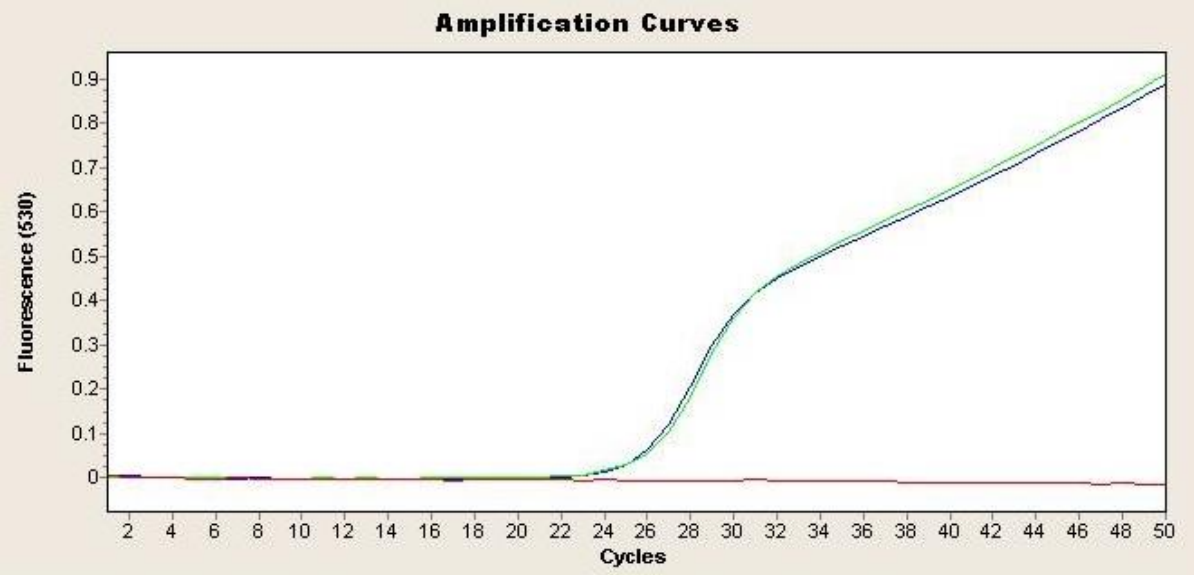

Figure 9: *Two CCHFV isolates amplification curves from A549 cell line and negative control. Cp values are 24.17 and 24.58.*

TCID50 assay was used for titer determination. The percentage of infected cells was observed with immunofluorescence microscopy and recorded for each virus dilution. The appearance of viral plaques was seen at higher dilution levels, while at lower dilution the vast majority of cells have become infected (Figure 10). Infected wells number results were used to mathematically calculate a TCID50 result with the Spearman-Karber method. During our experiments, a concentration of 2.15×10^4 TCID50/ml CCHFV stock was used.

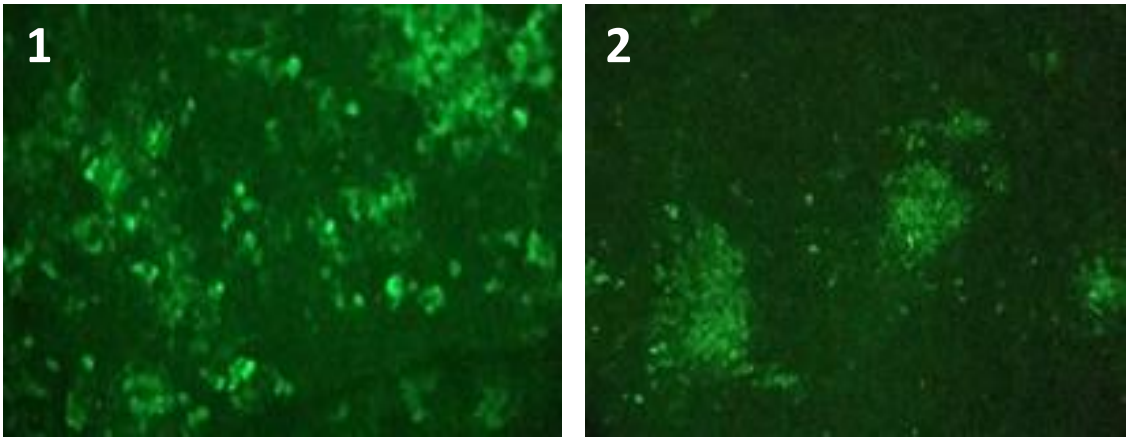

Figure 10: *(1) At lower dilution (10^{-1} dilution) the vast majority of cells were infected (100x zoom) (2) while the appearance of viral plaques was seen at higher dilution levels (10^{-4} dilution) (10x zoom).*

4.1.2 Recombinant nucleoprotein production

As the basis of our serological screening and mice immunization, we decided to utilize a recombinant nucleocapsid protein of CCHFV. Since CCHFV nucleoprotein production was already part of our previous research [48], we utilized the finished construct ("pET28a(+)-CCHFV" transformed into *E. coli)* and protocol during our research. Despite bacterial systems with protein expression is relatively easy and cost-effective, it has its shortcomings regarding the quality and quantity of the protein of interest.

Bacterial Protein Extraction Reagent® (B-PER, Thermo Scientific) was used to resuspend induced bacterial cells. Following cell exploration, the recombinant nucleoprotein was characterized by polyacrylamide gel electrophoresis. 55 kDa-sized recombinant nucleoprotein of CCHFV was revealed (Figure 11).

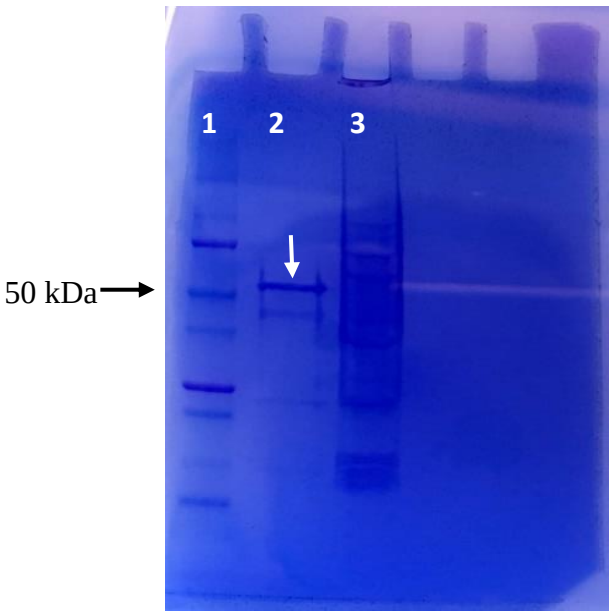

Figure 11: *SDS-PAGE image of purified CCHFV nucleocapsid protein with Brillant Blue. (1) Protein ladder (2) purified CCHFV nucleocapsid protein is marked with a white arrow at 55 kDa (3) B-PER supernatant with debris proteins obtained during purification.*

The recombinant nucleoprotein of CCHFV was validated by Western-blot. Immunized mice sera (4.1.3 Mice immunization) were applied 1:100 dilution as the primary antibody and HRP-conjugated rabbit anti-mouse IgG antibody (Dako) was used as a secondary antibody in a 1:800 dilution. CCHFV recombinant nucleoprotein was shown as a strong signal at 50 kDa. Weak protein signals with lower than 50 kDa were considered as being incomplete proteins produced during bacterial expression. This is presumed since protein bands below 50 kDa are not present in untransformed cells or cells containing vectors without insert, so it is assumed these are incomplete fragments generated from the insert. Furthermore, incomplete fragments were not labeled with a His-tag, as evidenced by the disappearance of unlabeled proteins following column purification (Figure 12/A).

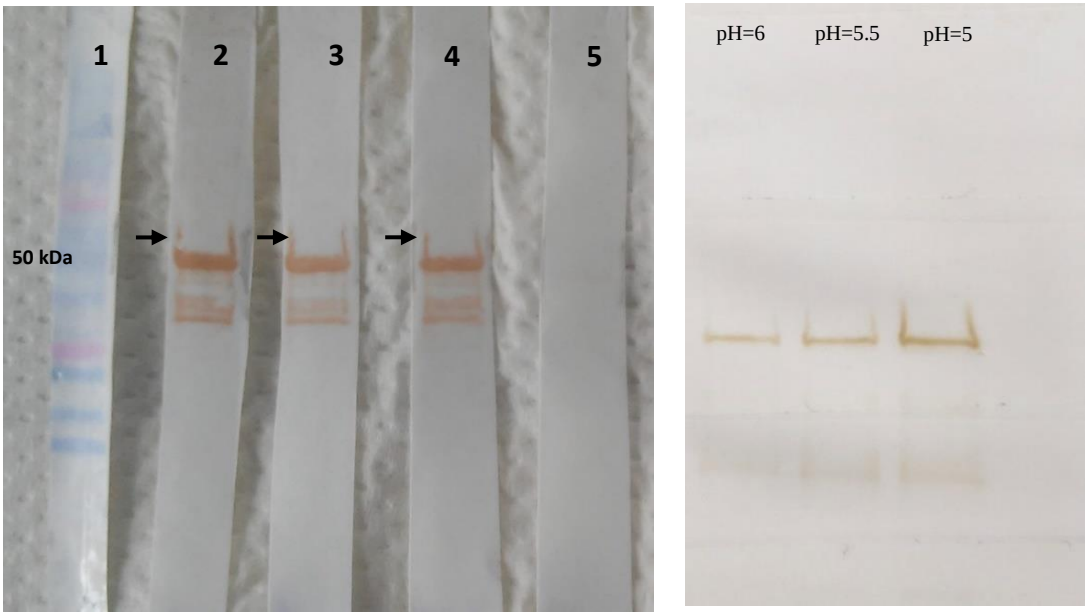

Figure 12: *Western-blot assays of recombinant CCHFV nucleoprotein. A) Recombinant nucleoprotein after bacterial protein extraction (1) Protein ladder (2-4) CCHFV nucleocapsid protein (marked with black arrow) prior to HIS Select HF Nickel Affinity Gel column purification (5) negative control. B) CCHFV recombinant nucleoprotein was superbly eluted at pH=5, pH=5.5, and pH=6 during HIS Select HF Nickel Affinity Gel column purification.*

Protein purification was performed under denaturing conditions, utilizing HIS Select HF Nickel Affinity Gel column (Sigma Aldrich). Following purification, WB was performed due to another validation. Immunized mice sera were applied 1:100 dilution as the primary antibody and HRP-conjugated rabbit anti-mouse IgG antibody (Dako) was used as a secondary antibody in a 1:800 dilution. CCHFV recombinant nucleoprotein was eluted most well at pH=5, pH=5.5, and pH=6 (Figure 12/B). Following purification, concentrations were measured as 89 µg/ml for CCHFV nucleoprotein.

4.1.3 Mice immunization

At the beginning of our experiment, no antibody against CCHFV was available on the market. Therefore we have to produce the necessary antibodies for staining. Three mice were immunized while one mouse was used as a control.

Immunized mice sera were tested by WB against recombinant CCHFV protein. ECL staining Western-blotting luminol reagent (Santa Cruz Biotechnology) was also used during WB as a secondary antibody in addition to an HRP-conjugated rabbit anti-mouse IgG antibody (Dako) (Figure 13).

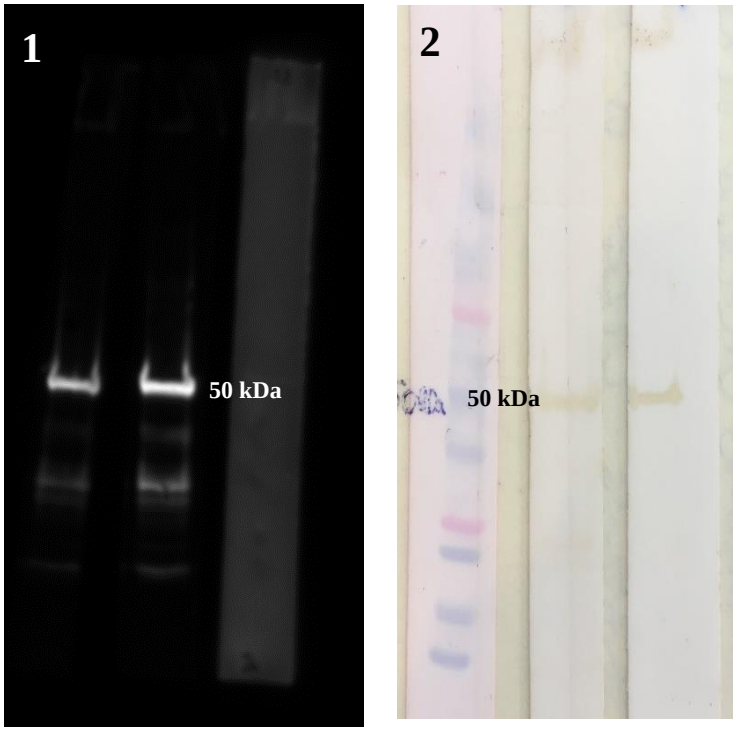

Figure 13: *Recombinant CCHFV nucleoprotein staining. Immunized mice sera were used as a primary antibody. HRP-conjugated rabbit anti-mouse IgG antibody (Dako) was used as the secondary antibody (1) ECL staining. (2) DAB color development staining.*

4.2 Serological surveillance results

During the serological surveillance, out of the 3207 collected serum samples, 2085 were selected for CCHFV antibody detection study. In the case of animals with multiple serum samples, only the samples taken the latest month were used, due to the persistence of antibody titer following infection.

We tested all 2085 individual rodents, dating from 2011 through 2013. Captured animals were categorized into four species: 1439 yellow-necked mice (*Apodemus flavicollis*), 448 bank voles (*Myodes glareolus*), 156 wood mice (*A. sylvaticus*), and 42 striped field mice (*A. agrarius*) (Table 3). Out of the four tested species, *A. flavicollis* was captured more significantly frequently in the span of the three-year-long screening ($\chi 2$=301.61; df=1; $p < 2.2\times10-16$).

In 2012, the number of specimens nearly doubled compared with 2011, with a significant difference ($\chi 2$=125.13; df=1; $p <2.2\times10-16$), while in 2013 the number of specimens was significantly lower compared to the previous two years ($\chi 2$=616.02; df=1; $p < 2.2\times10-16$).

Serological evidence of CCHFV was determined using two aspects of serologic assays: DBA pre-screening and IFA confirmation. Twenty out of 2085 (0.96%) tested rodents were positive for CCHFV IgG antibody using both methods (Figure 14).

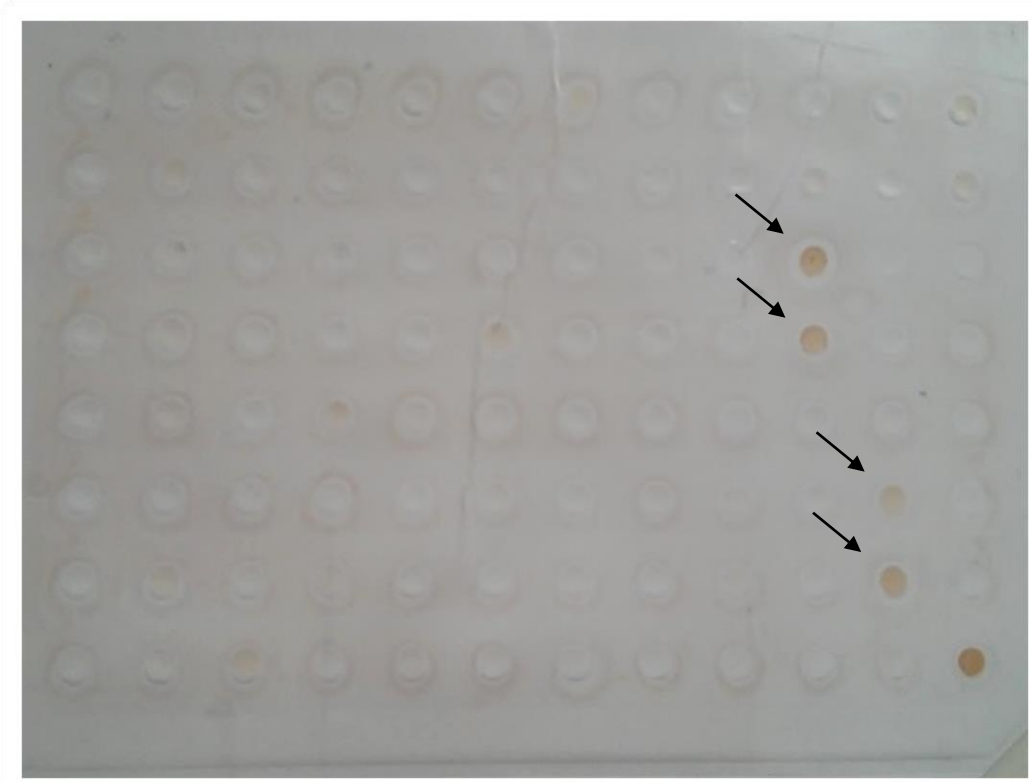

Figure 14: *Dot-blot assay color developed membrane. Right bottom: positive control. Four positive rodent serum samples were marked with arrows.*

During the IFA, the positive cell control, which stained primer antibodies as immunized mouse serum showed strong green fluorescence, while negative cell control failed to exhibit any specific staining. Thus, the reaction is not the result of non-specific binding between the cell and the antibody. Additionally, during the screening of wild rodent samples, a clearly distinct positive signal was obtained (Figure 15).

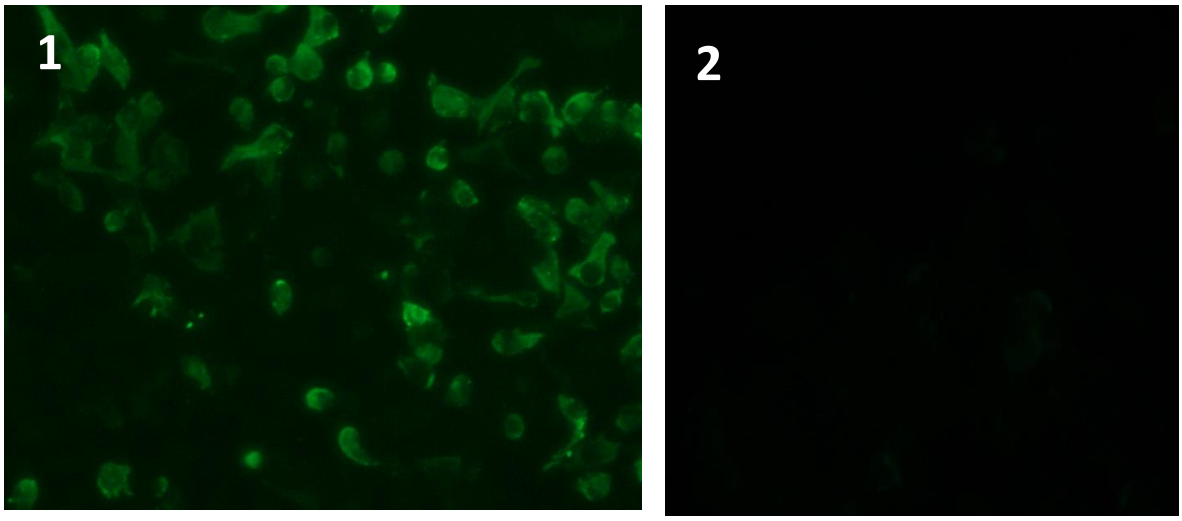

Figure 15: *Immunofluorescent assay: the positive cell control (1), which stained primer antibodies as immunized mouse serum showed strong green fluorescence, while negative cell control (2) failed to exhibit any specific staining (200x zoom).*

Out of the twenty CCHFV antibody-positive rodent sera samples, eighteen were *A. flavicollis*. One positive sample originated from *A. agrarius* and one from *M. glareolus*. None of the *A. sylvaticus* samples demonstrated seropositivity. Total seroprevalence per species was as follows: 1.25% (18/1439) in *A. flavicollis*, 0.22% (1/448) in *M. glareolus*, and 0.24% (1/42) in *A. agrarius*.

In 2011, five out of 684 rodent samples (0.73%) were positive. All five rodent sera originated from *A. flavicollis*. In 2012, ten out of 1165 rodent samples (0.09%) were positive against CCHFV antibodies. Out of ten positive rodent samples, eight sera originated from *A. flavicollis*, one from *A. agrarius*, and one from *M. glareolus*. In 2013, five out of 236 rodent samples (2.12%) were positive. Both positive rodent sera originated from *A. flavicollis* (Table 3).

Table 3: *Seroprevalence of tested rodents in south-western Hungary from 2011 to 2013.*

		Apodemus flavicollis			***Apodemus agrarius***			***Myodes glareolus***			***Apodemus sylvaticus***					Total seroprevalence
		Tested (n)	Positive (n)	%	Tested (n)	Positive (n)	%	Tested (n)	Positive (n)	%	Tested (n)	Positive (n)	%	Total tested	Total positive	
2011	March	19	1	5.3	0	0	0.0	9	0	0.0	3	0	0.0			
	April	91	1	1.3	1	0	0.0	9	0	0.0	9	0	0.0			
	May	95	0	0.0	0	0	0.0	5	0	0.0	9	0	0.0			
	June	112	0	0.0	1	0	0.0	1	0	0.0	18	0	0.0			
	July	150	3	2.0	5	0	0.0	18	0	0.0	19	0	0.0			
	August	58	0	0.0	8	0	0.0	25	0	0.0	3	0	0.0			
	September	12	0	0.0	0	0	0.0	4	0	0.0	0	0	0.0			
	Subtotal	**537**	**5**	**0.9**	**15**	**0**	**0.0**	**71**	**0**	**0.0**	**61**	**0**	**0.0**	**684**	**5**	**0.7%**
2012	March	51	0	0.0	3	0	0.0	7	0	0.0	6	0	0.0			
	April	62	1	1.6	6	0	0.0	22	0	0.0	10	0	0.0			
	May	95	0	0.0	9	0	0.0	17	0	0.0	7	0	0.0			
	June	99	1	1.0	2	0	0.0	47	0	0.0	6	0	0.0			
	July	65	1	1.5	3	1	33.3	31	0	0.0	7	0	0.0			
	August	167	5	2.9	0	0	0.0	57	1	1.8	24	0	0.0			
	September	85	0	0.0	1	0	0.0	92	0	0.0	10	0	0.0			
	October	113	0	0.0	1	0	0.0	55	0	0.0	5	0	0.0			
	Subtotal	**737**	**8**	**1.1**	**25**	**1**	**4.0**	**328**	**1**	**0.3**	**75**	**0**	**0.0**	**1165**	**10**	**0.1%**
2013	March	16	1	6.3	0	0	0.0	13	0	0.0	5	0	0.0			
	April	8	1	12.5	0	0	0.0	15	0	0.0	2	0	0.0			
	May	13	0	0.0	0	0	0.0	7	0	0.0	1	0	0.0			
	June	28	0	0.0	1	0	0.0	5	0	0.0	3	0	0.0			
	July	30	1	0.3	1	0	0.0	1	0	0.0	1	0	0.0			
	August	24	1	4.2	0	0	0.0	5	0	0.0	2	0	0.0			
	September	18	0	0.0	0	0	0.0	2	0	0.0	0	0	0.0			
	October	28	0	0.0	0	0	0.0	1	0	0.0	6	0	0.0			
	Subtotal	**165**	**5**	**3.0**	**2**	**0**	**0.0**	**49**	**0**	**0.0**	**20**	**0**	**0.0**	**236**	**5**	**2.1%**
	Total	**1439**	**18**	**1.3**	**42**	**1**	**0.2**	**448**	**1**	**0.2**	**156**	**0**	**0.0**	**2085**	**20**	**0.9%**

The sexual distribution of all tested and seropositive rodents was determined. During these three years, the ratio of males to females for all tested rodents was approximately equal (994:1087) and the seroprevalence also depicted this phenomenon (9:11).

Two outstanding seasons were observed during the three-year research era. Seropositivity was revealed in the spring (March and April) and during the summer (June, July and August).

4.3 RNA interference experiments results

In our study, fifteen siRNAs were designed and synthesized to test the inhibitory activity *in vitro* on CCHFV replication and target the mRNAs produced by S, M and L segments. We detected the high inhibitory effect of S (siS2), M (siM1), and L (siL3, siL4) segment-specific siRNAs. We experienced siRNAs inhibited CCHFV replication in a different efficiency and a dose-dependent manner.

4.3.1 Cytotoxicity tests

During the experiments, two different types of cell viability tests were used: light microscopic observation and luminescence cytotoxicity measurement.

The siRNAs treatment can cause visual cytopathogenic effects (CPEs) and affect viral growth, therefore we performed light microscopic observation to evaluate cell growth and viability. First, we had to find the appropriate siRNA concentration deemed effective in inhibiting CCHFV replication yet not toxic to the cells. In our cytotoxicity experiments, following three days of siRNAs transfection, the cell number per well was observed and compared to non-transfected cells by manual counting using a hematocytometer. In these experiments, we did not detect the cytotoxic effect of siRNAs on A549 cells at any lower concentrations used. However, a high concentration of siRNAs caused cell morphology changes and cell death.

In addition to morphological observation with light microscopy, luminescence cytotoxicity measurements (Promega – Cell Titer Glo Luminescent assay) were used. The cytotoxic concentration of the extracts causes death to 50% (CC50%) of viable cells in the host. The CC50 was calculated using GraphPadPrism version 8.00 software (Graph Pad Software, San Diego California, USA) for non-linear regression (Figure 16). In most cases, during cell viability tests, results observed microscopically were the same as the luminescence cytotoxicity measurements. Compared to the cell control and

transfection reagent control (Lipofectamine RNAiMax transfection reagent was added to A549 cells) mean cytotoxicity values elicited nearly identical values and showed no cell cytotoxicity in the luminescence cytotoxicity measurements.

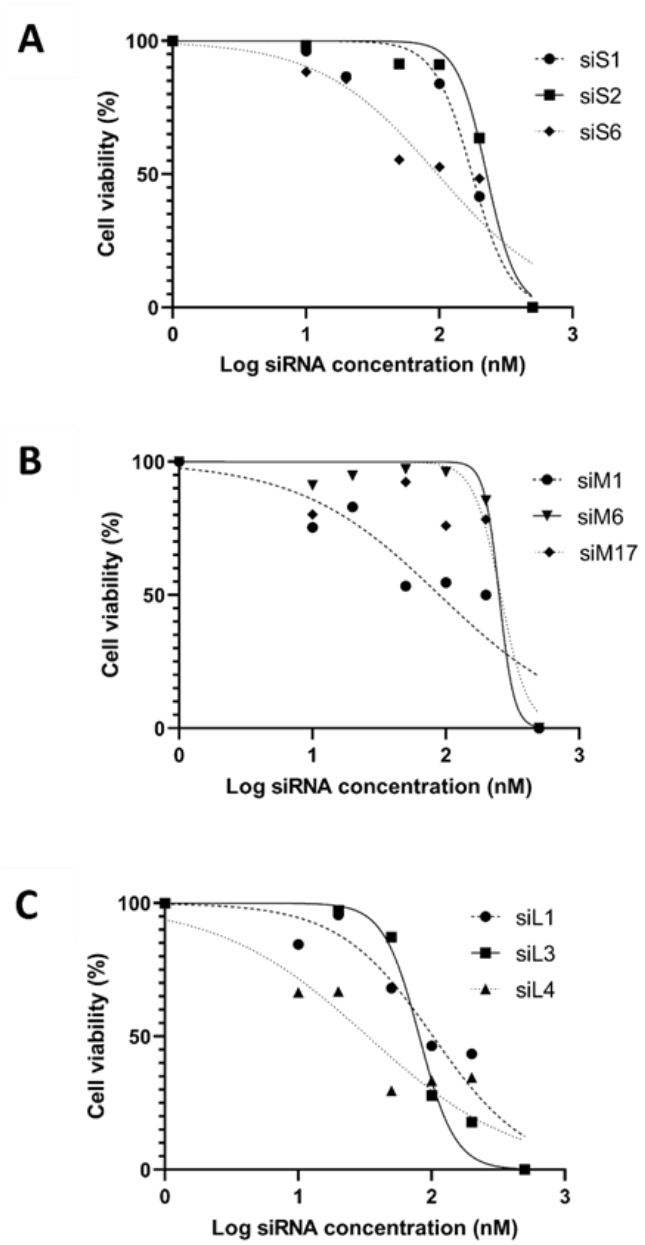

Figure 16. *The graphs depicts cell viability assay results using GraphPadPrism software.* ***(a)*** *Represents the effect of different concentrations of S segment siRNAs,* ***(b)*** *M segment siRNAs, and* ***(c)*** *the L segment siRNAs. Calculated CC50 and R squared data are represented in Table 4. The horizontal axis represents the logarithmized, different concentrations of siRNAs (nM) and the vertical axis represents the cell viability (%).*

In the case of siRNAs against the CCHFV S segment, the results were the same with the cell viability tests: CC50 was observed around 100 to 200 nM in every siRNAs against the S segment ($CC50_{siS1}$=177.7 nM; $CC50_{siS2}$=246.7 nM, $CC50_{siS6}$=106.5 nM). The highest S segment siRNA (siS2) CC50 value was 246.7 nM which was calculated using GrapPhadPrism version 8.00 software. CCHFV M segment siRNAs were proven to be non-toxic for the A549 cells at or about 100 to 300 nM concentration ($CC50_{siM1}$=99.84 nM; $CC50_{siM6}$=316.8 nM, $CC50_{siM17}$=298.8 nM). The highest M segment siRNA (siM6) CC50 value was extremely high (316.8 nM) compared to other siRNAs. In the case of siRNAs against the CCHFV L segment results were the same as the cell viability tests. The L segment siRNAs (siL3, siL4) CC50 values were 80.92 and 54.29 nM, respectively. SiL1 siRNA CC50 value was 109.7 nM. However, siRNAs against all of the segments were used in a maximum of 50 nM concentration due to comparability. The highest concentration used during experiments was determined by the lowest CC50 value of siRNAs (Table 4).

***Table 4**: Calculated CC50 values by GraphPadPrism software during cell viability assay.*

	siS1	**siS2**	**siS6**	**siM1**	**siM6**	**siM17**	**siL1**	**siL3**	**siL4**
CC50 (nM)	177.7	246.7	106.5	99.84	316.8	298.8	109.7	80.92	54.29
R squared	0.9758	0.9921	0.9376	0.8961	0.9842	0.8835	0.9334	0.9782	0.9038

In summarizing the cell viability results, the minimum concentration of siRNAs was set to 10 nM, and the maximum concentration to 50 nM in the case of every segment during our experiments. The subtle differences found between the cytotoxicity tests indicate the use of microscopic observation alone is not sufficient enough to detect cell viability and specify the appropriate concentration of siRNAs.

4.3.2 Transfection efficiency

During our study, transfection efficiency was calculated as the ratio between the numbers of viable siTOX-transfected cells versus non-transfected cells. In our experiments, we experienced an average of 80% transfection efficiency. Mock transfection is a transfection without siRNAs to control the potential effect of

transfection reagent (Lipofectamine RNAiMax) on the cells. In our experiments, the transfection reagent was not toxic for A549 cells (Figure 17).

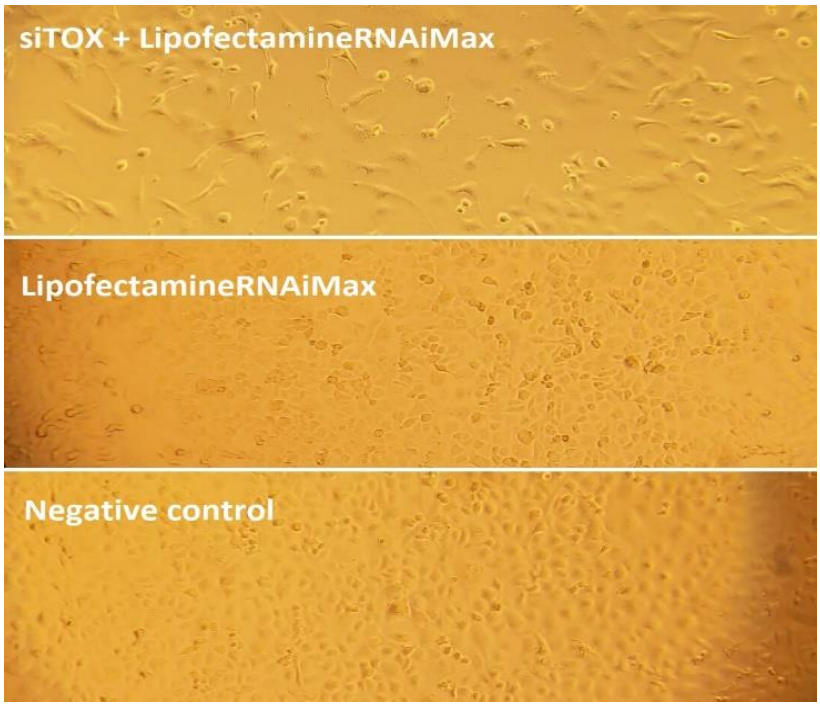

Figure 17: *Transfection efficiency test (100x zoom). From top to the bottom: (1) siTOX transfecting control with LipofectamineRNAiMax: transfected siTOX which is transfected successfully entered to the cell caused apoptotic cell death, (2) mock transfection (LipofectamineRNAiMax without siRNAs): no cytotoxic effect was experienced compared to (3) negative cell control.*

4.3.3 Inhibition of CCHFV replication using segment-specific siRNAs

qRT-PCR TaqMan assay was performed as pre-screening due to its large sample size, cost and time effectiveness. The siRNAs, which showed promising inhibitory effect against CCHFV based on qRT-PCR results, were chosen for further experimentation. Out of the fifteen siRNAs that were designed against CCHFV, nine were selected. During the pre-screening process, siRNAs were used in 10 nM and 200 nM concentrations. Following siRNAs transfection and virus infection, nucleic acid

extraction and qRT-PCR were performed. Taking into account the measurement results, we selected nine siRNAs considered to be effective according to the differences in the Cp values. In several cases, we found a difference of more than four Cp values (siM17, siL3), and we found a difference of almost four Cp values (siS2, siM1) (Figure 18). However, we also selected some siRNAs with a Cp value of around 1-1.5 (siS1, siS6, siM6, siL1, siL4) to see by performing a higher replication number and using different siRNA concentrations of how these siRNAs act. We did not select those siRNAs whose Cp value did not reach one (siS3, siS5, siM3, siM5, siL8, siL33).

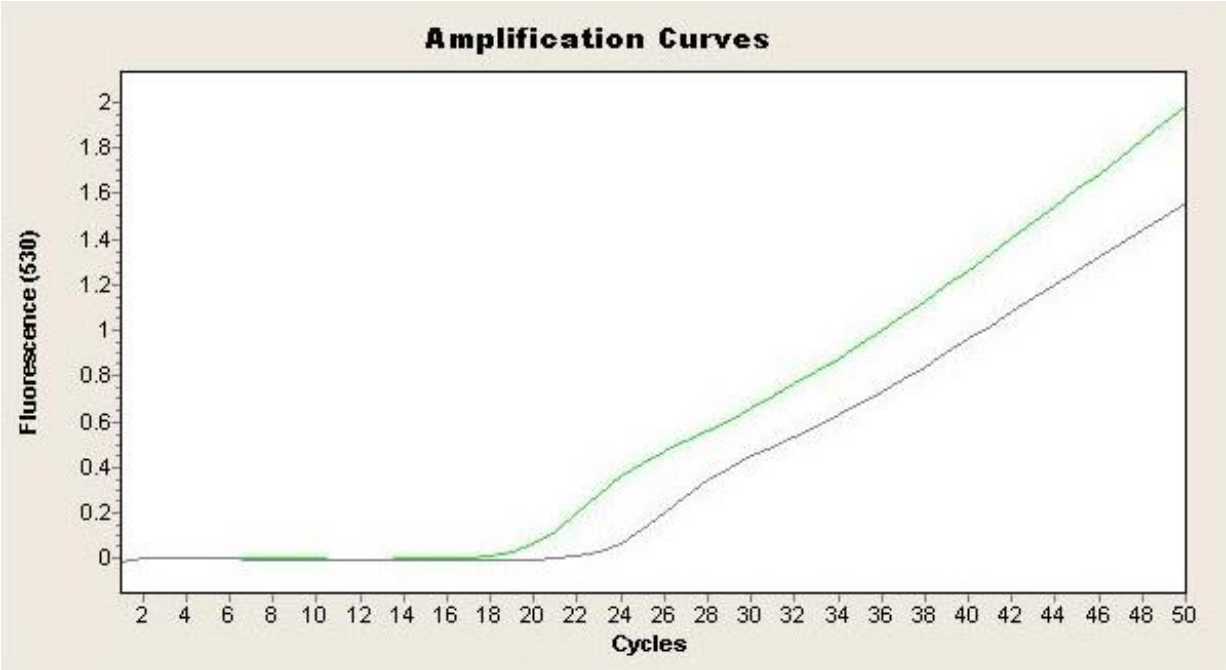

Figure 18: *siS2 Cp values decrease (3.71) was observed during CCHFV RNA amplification. The siRNA was used in two different concentrations: 10 nM and 200 nM.*

In our further RNA interference experiments, three siRNAs for every segment of CCHFV (siS1, siS2, siS6, siM1, siM6, siM17, siL1, siL3, and siL4) were used (Figure 19/A).

Based on the ddPCR results, a high and significant copy number decrease was detected in the case of several siRNAs (siS2, siM1, siL3, and siL4).

As depicted in Figure 19/B, when siS2 was used at 50 nM concentration, it has strong and significantly inhibited CCHFV replication compared to the positive control ($P<0.001$). Among siRNAs against CCHFV S segment, siS2 was the most efficient inhibitory siRNA. Furthermore, siS6 has shown a moderate yet significant inhibitory effect in CCHFV replication ($P<0.01$). In contrast, the significant antiviral inhibitory effect of siS1 at 10 nM concentration was not detected.

Among the various siRNAs which were designed for the M segment, siM1 had a strong and significant antiviral activity at 50 nM concentration ($P<0.001$). Moreover, siM6 has also reflected a CCHFV inhibitory effect at the medium level ($P<0.001$) at 10 nM concentration yet strong and significant antiviral activity at 50 nM concentration ($P<0.001$). In contrast, siM17 has not inhibited CCHFV replication significantly, whether at 10 nM or 50 nM concentrations.

In the case of the L segment, when siL4 was used at 10 nM and 50 nM concentrations, it strongly inhibited CCHFV replication compared to the positive control ($P<0.001$). SiL3 has also shown significant, high activities on CCHFV replication at 50 nM concentration. SiL1 has shown medium inhibitory effect at 10 nM and 50 nM concentrations (Figure 19/B).

We compared the amount of inhibitory effect of different siRNAs to the positive control. We did not use ON-TARGET plus non-targeting siRNA pool as a benchmark since it exhibits a result very similar to the positive control. Thus the positive control is suitable for comparison with the inhibitory effect of the siRNAs (Figure 19/C). The difference in copy number scale between experiments is given by the separate experiments and the degrees of dilution.

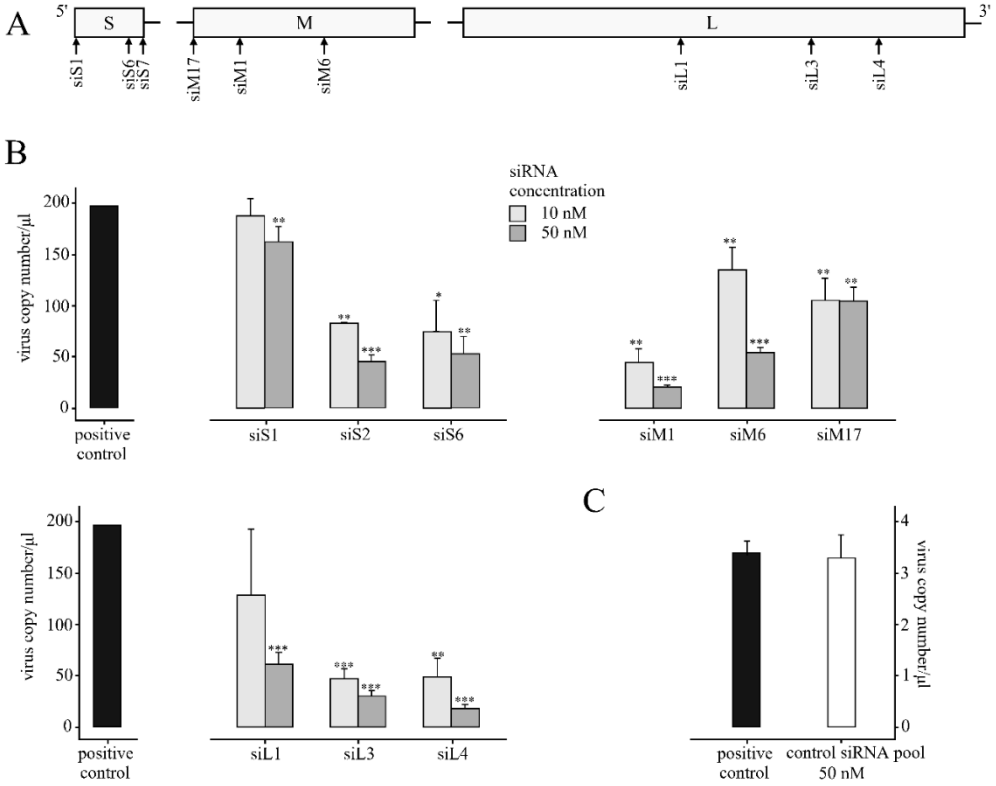

Figure 19: *A549 cells were transfected with siRNAs which were designed for CCHFV S, M and L segments in different concentrations (10 nM, 50 nM). Following transfection, cells were infected with CCHFV at a MOI of 0.1. Three biological replicates of siRNA inhibited CCHFV samples were used and the positive control was used in three biological and three technical repeats. The virus copy number was determined following seventy-two hours by RT-ddPCR.* ***(a)*** *CCHFV schematic gene map is containing designed CCHFV-specific siRNAs site;* ***(b)*** *Inhibitory effect of siRNAs against CCHFV: the horizontal axis represents the virus copy number/µl and the vertical axis represents the positive control and designed siRNAs. Student's t-tests were significant if *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$. Error bars represent the standard deviation (SD) of the means for three independent experiments.* ***(c)*** *Positive control and ON-TARGET plus non-target siRNA pool comparison: there is no significant difference between them by Student's t-tests. The horizontal axis represents the virus copy number/µl and the vertical axis represents the positive control and non-targeting control siRNA pool. Error bars represent the standard deviation (SD) of the means regarding the three independent experiments.*

At least one highly inhibitory siRNA was found in the case of every segment. The siRNAs designed for the S segment: the siS2 has shown an efficient decrease in the virus copy number at nearly 77% at 50 nM concentration. In the case of M segments siRNAs, siM1 has decreased the virus copy number by nearly 90% at 50 nM concentration. Among siRNAs designed to L segment, the most effectively siL4 has affected CCHFV replication (decrease by nearly 91%) at 50 nM concentration, much like siM1. The inhibitory effect against CCHFV was not caused by siS1 and siM17.

QuantaSofts' RT-ddPCR raw fluorescence readouts have shown negative and positive controls in Figure 20. A negative droplet population was shown by the negative control sample without any positive droplets. The positive control sample has appeared as a massive positive droplet population above the threshold level. In the case of the positive control sample, the positive droplet "rain" was caused by the high concentration of CCHFV and appeared as a background signal. The concentration-dependent high inhibitory effect was shown by siS2 and siM1. At 10 nM concentration, the positive droplet number was high in case of siS2, however, at 50 nM concentration, the positive droplet number was decreased. SiM1 acted similarly to siS2: high inhibitory effect was shown at 50 nM concentration. A medium inhibitory effect against CCHFV replication was presented by SiM6. The positive droplet number decreased moderately from 10 nM to 50 nM compared to siS2 and siM1 events. In the case of siM17, the significant inhibitory effect was not detected.

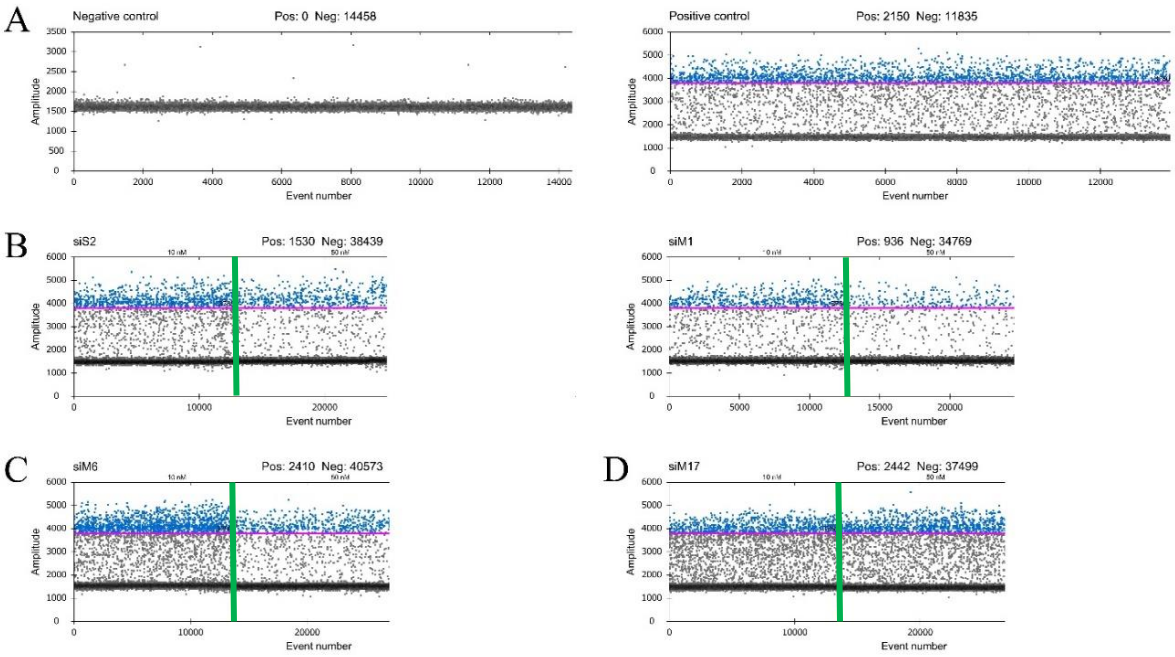

Figure 20: *QuantaSofts' RT-ddPCR fluorescent readouts. The horizontal axis represents the event number and the vertical axis represents the fluorescence amplitude in the FAM channel. The strict threshold line (pink line) was set for every sample at 3780 amplitude. Positive droplets were represented in blue and negative droplets were represented in grey.* ***(a)*** *The negative control sample was shown as the negative droplet population without positive droplets, the positive control sample was shown as the extensive positive droplet population;* ***(b)*** *siS2 and siM1 respectively were shown a concentration-dependent high inhibitory effect, different concentrations (10 nM, 50 nM) were separated with a green line;* ***(c)*** *siM6 has shown a concentration-dependent medium inhibitory effect at different concentrations (10 nM, 50 nM) which were separated using a green line;* ***(d)*** *siM17 has shown low inhibitory effect against CCHFV at different concentrations (10 nM, 50 nM) which were separated using a green line.*

5. DISCUSSION

5.1 Discussion of serological surveillance results

The current study reports data regarding CCHFV IgG antibody prevalence in four rodent species throughout Hungary, beginning in 2011 and extending through 2013.

The dominant species in the study area include the yellow-necked mouse (*Apodemus flavicollis*), the striped field mouse (*Apodemus agrarius*), the wood mouse (*Apodemus sylvaticus*), and the bank vole (*Myodes glareolus*). Among these rodents, *A. flavicollis* is the most predominant species at the study site. Our data acquired over the three years corroborate this fact since the majority of our samples originated from this species. There were also a large number of *M. glareolus*, especially in 2012, while *A. agrarius* and *A. sylvaticus* occurred in smaller numbers each year. There are a plethora of studies in support of the fact in which rodents are likely the primary distributors or hosts of many viruses throughout Hungary [81], [82], [89]. Due to the inherent risk among rodents undergoing exposure to ticks, they may also be one of the important carriers of the CCHFV, or even other nairoviruses.

In this study, the rodent sample numbers nearly doubled between 2011 and 2012. Density was the highest in 2012. Factors affecting rodent population fluctuations are well documented and includes various driver mechanisms, such as predators, climate, weather and source of food [90]. From 2012–2013, the number of samples decreased by nearly 80%. It is possible, in which the population experienced a breakdown and entered a phase of collapse.

In 2011, five out of 684 rodent samples (0.73%) were positive. All five rodent sera originated from *A. flavicollis*. In 2012, ten out of 1165 rodent samples (0.09%) were positive against CCHFV antibodies. Out of ten positive rodent samples, eight sera originated from *A. flavicollis*, one from *A. agrarius*, and one from *M. glareolus*. In 2013, five out of 236 rodent samples (2.12%) were positive. Both positive rodent sera originated from *A. flavicollis.* Out of the twenty CCHFV antibody-positive rodent sera samples, eighteen were *A. flavicollis*. One positive sample originated from *A. agrarius* and one from *M. glareolus*. None of the *A. sylvaticus* samples demonstrated seropositivity. Total seroprevalence per species was as follows: 1.25% (18/1439) in *A. flavicollis*, 0.22% (1/448) in *M. glareolus*, and 0.24% (1/42) in *A. agrarius*. To the best of our knowledge, no other Central European serological study is available which tested CCHFV antibodies in rodent populations. However, the seroprevalence data which we

obtained can be placed among the data tested between other animals and humans. In this study, we experienced 0.96% total seroprevalence during the three years. In Hungary, Magyar and colleagues experienced 0.44% seroprevalence between humans but they have not obtained information about the profession of the donors and risk of exposure to infected ticks or animals [91]. In this dissertation, we measured a slightly higher value (0.96%), which could be explained by the higher exposure of rodents to ticks compared to humans. Most studies also showed low or moderate values of seroprevalence in Central Europe [92], [93]. In Eastern and Southeastern Europe with several endemic countries, seroprevalence is higher than in the Central European region [19]. Sidira and colleagues in Greece found approximately 3.5% seroprevalence in the general population and high seroprevalence (up to 15%) in people working with animals in mountainous areas of the country [26]. Moreover, northeast Turkey is classically described as highly endemic for CCHFV [94]. The Western European region had the lowest seroprevalence values for CCHFV [95]. In Spain, Arteaga and colleagues found 0.58–1.16% seroprevalence in healthy blood donors [96].

In consideration of many pathogens, especially vector-borne microorganisms, transmission is generally seasonal. Rechav and colleagues found the immature stages of two species of *Hyalomma* ticks fed on hares and other small mammals. During these stages, they were infected with CCHFV. However, mostly, yet not exclusively, adult ticks transfer the virus to humans [97]. In our study, seropositivity was revealed in spring (March and April) and in summer (June, July and August).

Although *I. ricinus* ticks are, as of yet, an unsubstantiated species regarding CCHFV transmission, CCHFV was isolated from this tick species in suckling mice during the early 1970s in Hungary [47]. Based on this historical observation along with the previous serological studies, we hypothesized in which a new Central European CCHFV strain of the virus transmitted by *I. ricinus* may, in fact, exists today. *I. ricinus* larvae and nymphs in Hungary depict two peaks in seasonal activity, namely in April and July (spring and summer). On the other hand, preliminary experiments have shown small mammals are naturally parasitized by *I. ricinus* ticks throughout Hungary [50], [98].

Notably, DBA and IFA can lead to the detection of cross-reactive antibodies against CCHFV-like viruses, especially since we used polyclonal antibodies. Nevertheless, antibodies regarding related nairoviruses (Hazara virus and Dugbe virus) or the other viruses originating from the family *Bunyaviridae*, as seen in the Puumala

virus, have been shown not to cross-react with the recombinant nucleoprotein of CCHFV [25], [99]. Interestingly, the L and S segments of CCHFV which encode the RNA polymerase and nucleocapsid proteins, respectively, have a strong similarity to the Lassa virus (family *Arenaviridae*) [100]. However, we have no molecular evidence in support of viruses originating from the *Arenaviridae* family, such as the lymphocytic choriomeningitis (LCM) virus, originating from wild rodents throughout Hungary, thus far. The indirect IFA proved to be a more specific method. Based on the results of the current serologic investigation, we assume the presence of CCHFV throughout the region, yet, admittedly, further molecular-based studies are needed. Virus presence and re-emergence continue to be the main topics of national and international health security. The potential entry regarding CCHFV into new geographic areas is to be considered and requires appropriate and effective measures of surveillance to best acquire knowledge regarding virus ecology, transmission dynamics, and possible reservoir hosts and vectors in thus far, non-endemic regions.

5.2 Discussion of RNA interference against Crimean-Congo hemorrhagic fever virus results

Therapeutic options regarding the treatment of CCHFV infection are lacking, with the noticeable exception of ribavirin, which is currently recommended by the WHO. Nevertheless, novel and more sophisticated antiviral therapies against nairovirus infections are urgently needed. In the past few years, several studies have shown siRNAs have the potential to be operated as a specific therapeutic strategy against some viral infections [25], [68], [78], [101], [102]. However, most of these experiments are in the *in vitro* test phase, and translating RNAi in the clinic, as a conventional treatment option remains a pivotal challenge. In the case of *in vivo* therapies, one of the most difficult parts is efficiently and specifically delivering siRNA to target tissues and cells. Moreover, the poor cellular uptake of siRNAs in combination with rapid enzymatic degradation is limiting RNAi usage *in vivo* therapies. Due to the association of siRNAs with a non-target gene, an off-target effect can occur. The off-target effect is the other main limitation of *in vivo* applications. Additionally, different classes of siRNA chemically modifications can increase the efficiency of delivery. Fortunately, despite difficulties in virus entry, cytotoxicity and the stimulation of unspecific immune response researches evolved and reached *in vivo* experiments [103].

One of the main influencing factors in reference to RNAi experiments is the off-target effect, in which siRNAs do not bind to the target gene, but induce silencing in other non-targeted genes. Caffrey and colleagues studied the incidence of nonspecific targeting is likely dependent upon the concentration of the siRNA, with a higher concentration leading to a greater off-target effect [104]. Therefore, taking into account the results obtained from the cytotoxicity assay, we kept the concentration of siRNAs low, and we maximized it to 50 nM to minimalize off-target effects. Antiviral-response pathways, inducing the expression of antiviral-response genes are stimulated by high concentrations of siRNAs in the cells [105].

We evaluated the antiviral activity of siRNAs targeting the S (nucleoprotein), M (glycoproteins), and L (polymerase) transcripts of CCHFV for the first time *in vitro*. The siRNAs were designed for each CCHFV segment in an effort to find the most effective ones. We observed in which, among fifteen tested siRNAs, four of them (siS2, siM1, siL3, siL4) were capable of reducing CCHFV copy numbers by more than nearly 70% during in vitro infection studies, comparing to the positive control. However, strong inhibition of CCHFV replication (nearly 90%) was performed by only two siRNAs (siM1, siL4). The CCHFV RNA decrease was observed with RT-ddPCR, which does not completely demonstrate infectious virus particles yet the purpose of our experiment was to establish the ratios of CCHFV RNA decrease and effect/non-effect of siRNAs. The unusual ability of many siRNAs to inhibit the virus, contrary to previous studies, is due to the successful design and the high rate of transfection achieved.

Off-target effects occur when a siRNA is processed by the RNA-Induced Silencing Complex (RISC) and down-regulates unintended targets. These changes in gene expression can lead to false-positive results. Therefore, off-target effects caused by the incorporation of the passenger strand - instead of the antisense strand - of the siRNA into RISC can be avoided by designing the siRNA ends to favor RISC uptake by the intended active strand [106]. Moreover, siRNA sequences were subjected to a BLAST search against GenBank to minimalize off-target effects. In addition, ON-TARGET plus non-target siRNA pool was also used to exclude false results.

In the case of the CCHFV S segment protein, nucleoproteins play a central role in the regulation of viral replication. Nucleoprotein associated with genomic viral RNA to form RNPs and provided as a template for the polymerase. In the last few years, several homologous interferences have been described as the inhibition of S segment of other nairoviruses by siRNAs and suppression of viral replication [68], [70], [102]. Levin and

colleagues discovered the akabane virus (AKAV) infected Vero cells indicated more than 99% inhibition [102], while Chiang and colleagues described how siRNA against the S segment of the andes virus (ANDV) greatly reduced levels (>60%) of viral protein expression [70]. In the case of the hazara virus inhibition, the siRNAs which were designed against the S segment had a higher effect (up to 90%) than those targeting M and L segments [68]. Several experiments performed have shown the S segment of the genus *Orthobunyavirus* is the RNA interference prime target in arthropod cells [107], [108]. In our study, among those siRNAs designed against the S segment, siS2 has inhibited effectively CCHFV copy number. Our study is in agreement with previous works [68], [70] in which targeting the S segment by siRNAs can produce an effective inhibitory impact. Consequently, using the S segment as the target for silencing virus replication has proven to be an option for future therapeutics. Hereafter, using siRNAs together can have a superior effect against virus infections [102]. Our strategic plans include the combined and pooled use of designed siRNAs against CCHFV infection.

CCHFV glycoproteins (Gn, Gc) are involved in cell entry, initial binding, and fusion. However, the details of specific glycoprotein involvement remain unknown [52]. In contrast with other studies, a high inhibitory effect of siRNA (90%) was found against the M segment. Furthermore, Chiang and colleagues described in which viral glycoproteins are limiting factors for virus production and viral glycoproteins are detected mainly in the lysosome rather than on the cell surface in genus *Orthonairovirus* endothelial cells. In this study, reducing the glycoprotein levels with siRNA against the M segment had a greater impact on the virus copy number (decreased by nearly 90%) and release [70]. Moreover, the M segment is the most diverse genome regarding CCHFV. This diversity may come from how CCHFV uses the vectors and vertebrate hosts in different geographic ranges. Therefore, it is difficult to design general well-functioning inhibitory siRNAs for this segment and many studies found a lower inhibitory effect. Although glycoproteins encoded by the M gene are the most variable portion of the CCHF viruses, some functional domains of the glycoproteins are well conserved [52], [109].

In the case of CCHFV, the largest of the three segments labeled as the L segment, encodes an RNA-dependent RNA polymerase (RdRp) which is characterized by several conserved functional regions [52]. Moreover, next to nucleoprotein, L protein drives the processes of transcription and replication which occur in the cytoplasm during the viral replication cycle. Thus, targeting this segment is likely to be an exact strategy. We

found siL4 caused 90% reduction in the CCHFV copy number compared to the positive control.

Comprehensively, our results provide additional support for the use of RNA interference-based techniques in the development of antiviral drugs against CCHFV infections. To the best of our knowledge, this is the first study which used designed siRNAs against CCHFV replication *in vitro* and the first study to provide RNAi solution to all three genomic segments of a nairovirus in parallel. Currently, CCHFV constitutes a notable public health concern in our region, with significant geographic expansion in recent decades and growing epidemic potential [48], [49], [67]. One major limitation of our study is the lack of combinative experiments, however, it ideally projects future research directions. Combining efficient siRNAs may reveal their potential synergic inhibition effect. Accordingly, the threat of viral infection will increase in the coming years, hence, any kind of research project aimed at preventing and overcoming a possible infection may prove useful. Moreover, we wish to design time-dependent experiments which examine siRNAs efficiency before and after CCHFV infection, since they are required for *in vivo* experiments regarding the future. This study gives novel and important research results for one of WHOs prioritized emerging disease and constitutes a major step for future antiviral development efforts.

6. SUMMARY

An increasing number of emerging infectious diseases have been discovered in recent years which mostly have a viral origin. Approximately 75% of new emerging human infectious diseases are defined as zoonoses. Numerous studies focus upon the analysis of these infectious zoonoses, their risk factors, their prevention and cure and in the identification or their virus hosts. Molecular characteristics regarding viruses immensely contribute to the emergence of new types (e.g., high mutation rate, recombination and reassociation). Additionally, the intrinsic characteristics of pathogens, the spatial distribution and the lifestyle of the host also influence the emergence of infectious agents, survival and intensity. Environmental, climatic and urban factors can also affect the ecological attributes of these viruses.

Crimean-Congo hemorrhagic fever virus is characteristically, one of the most significant zoonotic agents. Its geographic range is the most extensive one among the medically important tick-borne viruses. Currently, there is no antiviral drug or vaccine available for CCHF. The development of effective treatments for the prevention of CCHFV caused disease is now a priority for both public health and biodefense agencies.

Due to the increasing spread of CCHFV worldwide and the lack of therapy, one of the main aims of this thesis was to obtain more information in reference to the CCHFV situation in Hungary by determining the seroprevalence of the rodent population. The other main aim of this thesis was to provide a basis for future antiviral therapies by the design of highly effective siRNAs which can inhibit CCHFV replication.

In this study, 20 out of 2085 tested rodents were positive for CCHFV IgG antibodies using DBA and IFA. We found 0.96% total seroprevalence between rodent populations in Hungary. The serological results are consistent with other studies seroprevalence values in Central Europe [19], [95]. Out of the 20 CCHFV antibody-positive rodent sera samples, 18 were from *A. flavicollis*. One positive sample originated from *A. agrarius* and one from *M. glareolus*. None of the *A. sylvaticus* samples demonstrated seropositivity.

In this dissertation, the ecological background, and the reliability of CCHFV was studied in Hungary among rodent populations. Based on the serological results, we assume the presence of CCHFV throughout the region, yet, admittedly, further

molecular-based studies are needed. Based on our results, we recommend an extended sero-surveillance study focusing on both animal and both human populations. Additionally, a systematic tick surveillance study in Hungary will also prove useful to estimate the distribution of ticks and investigate their potential role as vectors of CCHFV. Moreover, raising awareness of the growing CCHFV threat in risk populations (health care workers, shepherds, farmers, veterinarians, slaughterhouse workers, soldiers, campers, etc.) is also important.

While offering a practical aspect of the work, we tested the RNA interference as a novel, possible and rapidly evolving therapeutic solution as a viral replication inhibitor regarding future therapy. To our knowledge, this is the first study which makes use of designed siRNAs against CCHFV replication *in vitro* and the first study to provide a RNAi solution to all three genomic segments of a nairovirus. In consideration of our results, we observed from a pool of fifteen tested siRNAs, four of these (siS2, siM1, siL3, siL4) were capable of reducing CCHFV copy numbers by more than nearly 70% during *in vitro* infection studies, compared to the positive control. However, strong inhibition of CCHFV replication (nearly 90%) was performed by only two siRNAs (siM1, siL4).

An increased risk of viral infection is expected in the near future, so research projects aimed at preventing and overcoming a possible infection may prove vital.

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9. LIST OF PUBLICATIONS

9.1 Publications beyond thesis topic

Földes F, Madai M, Papp H, Kemenesi G, Zana B, Geiger L, Gombos K, Somogyi B, Bock-Marquette I, Jakab F Small interfering RNAs are highly effective inhibitors of Crimean-Congo hemorrhagic fever virus replication *in vitro* MOLECULES 25, 5771 (2020)

Földes F, Madai M, Németh V, Horváth Gy, Bock-Marquette I, Herczeg R, Jakab F Serologic survey of Crimean-Congo haemorrhagic fever virus infection among wild rodents in Hungary TICKS AND TICK-BORNE DISEASES S1877-959X(19)30089-5 (2019)

9.2 Oral and poster presentation beyond thesis topic

Földes F, Madai M, Papp H, Kemenesi G, Zana B, Geiger L, Gombos K, Jakab F Krími-kongói vérzéses láz vírus replikációjának gátlása siRNS-ekkel. XVIII. Szentágothai János Multidiszciplináris Konferencia és Hallgatói Verseny Absztrakt kötet XVIII. János Szentágothai Multidisciplinary Conference and Student Competition Book of Abstracts Pécs, Magyarország, János Szentágothai Scholastic Honorary Society, Faculty of Sciences, University of Pécs, 2020 pp. 19-20. 2 p.

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