REVEALING THE *IN VITRO* GROWTH-ADAPTIVE CHANGES OF CRIMEAN-CONGO HEMORRHAGIC FEVER VIRUS AND CONDUCTING THE FIRST COUNTRY-WIDE SEROSURVEILLANCE AMONG HUMANS AND ANIMALS IN HUNGARY

Ph.D. thesis

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1. Introduction

Crimean-Congo hemorrhagic fever is a widespread tick-born disease that can cause various symptoms including hemorrhagic manifestations in humans with a reported fatality rate of 5–40% among hospitalized patients. The number of annual cases and seroprevalence is increasing worldwide. The causative agent, the *Orthonairovirus haemorrhagiae* – previously known as Crimean-Congo hemorrhagic fever virus (CCHFV) – is endemic to the Balkan region, Eastern and Southern Europe, the Middle East, Africa and Asia. In correspondence with the geographical distribution of the virus, which coincides with the expanding spatial spread of the principal tick vectors, CCHFV is a genetically diverse pathogen. Three out of seven distinct lineages are circulating in Europe: the Europe-1, the Europe-2 and the Africa-3 lineages. Studies have identified over 30 tick species as potential virus carriers (*Hyalomma spp., Rhipicephalus spp., Ornithodoros spp., Dermacentor spp., Ixodes spp.* ticks, etc.). Due to global warming and the movement of migratory birds, the distribution of Mediterranean tick species has shifted further north in the previous decade, including Hungary. Although no human CCHFV cases has been reported in the country, based on vector presence and serological evidence among small vertebrates (rodents and brown hares), Hungary lies within high evidence consensus for potential CCHFV introduction and future human infection. As part of a successful One Health approach, sufficient information as part of a broad-range serosurveillance among

the human population and free-range indicator animals, such as cattle and sheep are crucial to better understand human disease and the outbreak potential of CCHFV. Due to the great public health risk, epidemic potential, and the lack of sufficient countermeasures, the World Health Organization identified CCHFV as one of the priority pathogens in the terms of diagnostics, therapeutics and vaccination development. Links between infectivity and mutagenesis for long-term virus conservation is a particularly important quality management issue in the case of highly variable single-stranded RNA viruses, such as CCHFV. Furthermore, genetic variations may lead to phenotypic changes regarding infectivity and viral fitness, thereby changing the characterized properties of the virus strain used in laboratory experiments. Although many different cell lines are used for *in vitro* propagation of CCHFV, the degree of mutations in the viral genome and the variation frequency in specific cell lines are still unknown.

2. Objectives

By incorporating the international standards and requirements into our CCHFV research, we set out the following goals in this thesis:

1. Phenotypic and genetic characterization of the CCHFV Afg09-2990 laboratory strain in a stable and controlled cellular environment, in order to
    i. describe virus growth kinetics in different cell lines;
    ii. determine the optimal conditions for virus propagation in different cell lines (multiplicity of infection, day to harvest, cell lines with high permissivity);
    iii. determine the degree of growth-induced phenotypic changes and genetic variations in the virus genome.
2. As part of the One Health concept, by using well-established and validated in-house and commercial serological assays, we aimed to perform the first broad-range CCHFV serological surveillance studies in Hungary among
    i. the general human population (healthy blood donors); and
    ii. free-range vertebrates as indicator animals (cattle and sheep).

3. Methods

The CCHFV Afg09-2990 strain was used in our experiments. All work with infectious virus was performed under BSL-4 containment at the National Biosafety Laboratory, National Center for Public Health and Pharmacy.

3.1. In vitro growth-induced adaptation to cell lines

To describe virus growth kinetics, four different cell lines susceptible for CCHFV (Vero E6, Vero, SW13 and BHK-21) were inoculated in three biological and three technical replicates by using three different multiplicity of infection values (MOI 0.005, 0.01 and 0.1). Cell culture supernatant of each sample was collected daily, until day 7 post inoculation. Based on virus growth, the optimal MOI and day post infection for harvesting the supernatant were determined and used for the subsequent (10 rounds of) passages, including 5 rounds of cross-passaging (P6 – P10) in different cell lines. Virus RNA copy number and infective titer per mL were determined by RT-qPCR and infective titration in SW13 cells by observing cytopathic effect in 96-well plates. Whole genome sequencing from the extracted viral RNA was performed by using the single-primer sequence-independent amplification (SISPA) protocol, followed by Illumina Nextera XT V2 library preparation and paired-end sequencing on the Illumina MiSeq platform. Kinetic curves were visualized in the GraphPad Prism 9.5.0 software. To analyze the effect of the different cell lines, the MOI and the passage number on the log virus growth, an ordinary two-way

analysis of variance was performed. Cell permissivity – described as the initial (d0) virus RNA copy number used for inoculation over the generation of 1 TCID50 per mL – was also determined during serial passaging. For the genome analysis, reference mapping of the quality trimmed reads was performed. Variants over 10% frequency were called and the distribution of nucleotide variants by segment and genome position was determined. Analysis of the nucleotide variants and manual annotation of high-frequency mutations over 40% frequency (consensus level) were performed and compared to the whole genome sequence of the initial virus stock.

3.2. Human and animal serosurveillance

As part of the retrospective human serosurveillance, serum samples taken from randomly selected blood donors between 2008 and 2017, provided by the Hungarian Blood Transfusion Service were screened for anti−CCHFV IgG antibodies. Donors aged between 18–65 years were enrolled from all 20 statistical regions of Hungary. Sample size was fitted to the Hungarian demographic data to reach 0.04% prevalence of the population. Samples (n = 2700) were tested by using in-house indirect immunofluorescent assay (IIFA) produced and validated at the National Biosafety Laboratory. IIFA–positive results were verified by using a commercially available recombinant nucleoprotein ELISA kit. For the descriptive analysis, the number of reactive samples and seroprevalence were determined for each statistical region by age and gender. Geographical distribution of seroprevalence was visualized by place of blood donation.

To perform the first broad-range animal serosurveillance in Hungary, blood specimens were tested taken from healthy free-range animals (cattle and sheep) as part of monitoring programs for various animal diseases by the Laboratory of Immunology of the Veterinary Diagnostic Directorate, National Food Chain Safety Office in 2017. In total, 1905 serum specimens were tested for the presence of anti−CCHFV IgG antibodies, comprising 1391 samples obtained from cattle and 514 samples from sheep. Samples were received from 13 statistical regions of Hungary. The sera of animals from the same district were combined into pools of a maximum of five samples. To screen the cattle, the Cow Crimean–Congo Hemorrhagic Fever Virus GPC IgG (CCHF-IgG) ELISA Kit was used. Samples of the positive or equivocal pools were tested further individually with the same ELISA kit and two types of immunofluorescent assays (the EUROIMMUN CCHFV Mosaic 2 IgG IIF assay and our in-house IIFA). To investigate the sheep serum pools, the in-house IIFA and the EUROIMMUN CCHFV Mosaic 2 IgG IIF assays were used. Among the cattle samples, those that showed reactive results in ELISA and both IIFA assays were considered positive, and among the sheep samples, positivity was concluded if reactivity was detected in both in-house and commercial IIFAs. Prevalence and geographic distribution were defined in all study groups in each statistical region and district.

4. Results

4.1. In vitro growth-induced adaptation to cell lines

The highest virus RNA copy number and TCID50 per mL was determined in SW13 at all three MOIs. Virus growth kinetics showed similar trends in BHK-21 cells as in SW13, however, without producing CPE in the cells. Similar kinetics were seen in Vero and Vero E6 cells at all MOIs. The maximum virus RNA copy number and TCID50 per mL increased to the same extent at all MOIs, but depended on the cell line. Consequently, the logarithmic increase in the virus growth was higher when inoculated at MOI 0.005 compared to the higher MOIs. According to the ordinary two-way ANOVA, the MOI and the cell line factors had significant effect on the logarithmic increase in virus RNA copy number, but the cell line factor had no effect on the logarithmic increase in the infective titer. Based on the results of the MOI-dependent virus growth kinetics, we chose MOI 0.005 for the subsequent (P2 – P10) passages. Permissivity of the cell lines for CCHFV was determined over serial passaging. The highest initial permissivity was found in SW13 cells (1.09E+02 copies/TCID50), however that reduced by 0.81 log by P10. The highest increase in permissivity (1.99 log on average) was seen when the virus was passaged in Vero cells, suggesting adaptation to Vero cells. The lowest initial permissivity was observed in BHK-21 (5.16E+03 copies/TCID50), however it increased with 1.30 log on average due to serial passaging.

4.2. Growth-induced genetic variations

During the study, we identified an overall number of 953 SNPs, including 256 unique mutations with frequency over 10%. In the S

segment, the average variant frequency (VF) increased from 11.12% (P1) to 19.61% (P10). In the M segment, the average VF was 13.93% (P1), and increased to 16.27% (in the range of 13.20% in SW13 and 19.17% in BHK-21) by P10, depending on the new cell line. In the L segment, the average VF showed strong increase from 16.27% (P1) to 39.23% (P10). In the S segment we found two individual nt variations with frequencies over 40%, of which one silent mutation is a probable consequence of adaptation to the BHK-21 cell line (I448I). In the M segment, most SNPs with frequency over 40% emerged when the virus was previously passaged in Vero cells (L276L, E594K, D1168G). These mutations might contribute to the enhanced permissivity of this cell line for CCHFV. In the L segment we identified 10 mutations that can be linked specifically to a cell line. Two nt variations were present in the original P0 sequence as ambiguities: A2279V in the RdRp region that further persisted in all cell lines with 12.06 – 100.0% frequency depending on the cell line, and E1093E that persisted further only when passaged in Vero cells. Mutations D618E in the zinc-finger domain, P899P in the NP-binding region and F2576F in the RdRp persisted throughout the long-term passaging when the virus was propagated in BHK-21. Mutations A1159T and T3667A emerged only when the virus was passaged in Vero, while N2025N, A3879V and R837S emerged Vero E6 and SW13-specifically. Nucleotide change preference among variants was also determined in all three genome segments. The most common high-frequency nt change in the S segment was T > C for all cell lines.

In the M segment, the most common nt changes were A > C and A > G. In the L segment, we identified mostly T > C and A > G changes.

4.3. Human serosurveillance

Altogether, 12 samples (0.44% seroprevalence) were positive for anti−CCHFV IgG antibodies after pre-screening by IIFA. Reactivity was verified by a rNP ELISA kit. Obtained results were concordant with the IIFA for eight samples and two samples were determined as borderline. The average age of blood donors with reactive results (n = 10) was 37 years (22–64 years). Among the ten positive samples detected, eight were taken from male donors. The calculated seroprevalence was 0.55% among male donors and 0.16% among female donors. The most affected age group was Group I (18 – 34 years) with a seroprevalence of 0.78%. Positive samples were identified in 8 statistical regions, with a prevalence of 0.20 – 2.97%. The western and central parts of the country were the most affected (Győr-Moson-Sopron, Vas, Veszprém, Somogy, and Fejér counties), together with Bács-Kiskun county in the South-Central part of Hungary. The highest seroprevalence was observed in Jász-Nagykun-Szolnok county in central Hungary, with two confirmed samples and one probable sample. The average prevalence in the affected statistical regions was 1.38%. Positive samples were identified in eight out of 73 blood donating locations.

4.4. Animal serosruveillance

During the first screening, six pools of cattle samples and one pool of sheep samples were reactive ($n = 3$) or equivocal ($n = 4$). Serum specimens of these pools were tested further individually. Among the cattle, a total of eight samples (from five pools) showed anti−CCHFV IgG reactivity in all three assays. Among the sheep, three reactive samples (with both IIFA assays) were found originated from a single sample pool. Among cattle, seropositive samples were identified in five districts from four statistical regions, one animal from the Csongrád district (Csongrád county), three animals from the Baja and Kiskunfélegyháza districts (Bács–Kiskun county), three animals from Csorna district (Győr–Moson–Sopron county), and one cow from Várpalota district (Veszprém county). Among sheep, we found three seropositive samples from the Kiskunfélegyháza district (Bács–Kiskun county). Overall, the most affected statistical region was Bács–Kiskun in the south–central part of Hungary, with an average seropositivity of 1.8%. Seropositivity among tested animals, including both cattle and sheep in the affected statistical regions was 2.33%. Regarding geographical distribution, our data obtained in the frame of the animal serosurveillance correlate with the seroprevalence among the general human population, and are also comparable with the historical reports of the primary vector, the *Hyalomma* ticks in Hungary.

5. Conclusions

Based on previously reported data, Hungary stands within high evidence consensus in the aspect of CCHFV transmission and circulation. To incorporate international recommendations and requirements into our CCHFV research, we studied phenotypic and genotypic changes in the CCHFV genome in a stable and controlled cellular environment. The methods used allowed us to identify mutations at low frequency arising across the entire viral genome, as expected for RNA viruses with a high error rate during replication. As changes in the viral genome and the emergence of new viral quasi-species can affect virus properties, such as pathogenicity, fitness, host-adaptation by evading immune response, enhanced host-cell permissivity, etc., genetic variations can have impact on the sensitivity and specificity of antiviral, vaccine efficacy and diagnostic assays as well. Virus growth kinetics was determined in four cell lines (Vero E6, Vero, SW13 and BHK-21) at different multiplicity of infection values. In the frame of this thesis:

- We established a mutation accumulation experiment by performing ten rounds of serial passaging and cross-passaging, followed by whole genome sequencing and analysis to map growth-adaptive signature mutations.
- We found that all four tested cell lines are susceptible for CCHFV, and permissivity increased due to serial passaging in Vero and BHK-21 cells.

- We determined that growth-induced mutations emerged in a cell-line specific manner, as the applied cell lines had significant effect on the mutation variant frequency, especially in the L segment.

By mapping growth-induced mutations, we were able to combine viral evolution in controlled laboratory settings, as well as phenotypic characterization of CCHFV.

As part of our research, we performed the first retrospective and systematic CCHFV serosurveys focusing on human and animal populations in Hungary covering a significant geographical area. Although the serum samples were taken before 2017, we obtained essential data on the CCHFV serological status in Hungary from a few years ago.

- In total, 2700 human and 1905 serum samples taken from free-range cattle and sheep were tested for the presence of anti−CCHFV IgG antibodies using a range of commercial in-house assays.
- We found a total of 10 reactive samples among humans (0.37%) and 11 reactive samples (0.58%) among animals comprising 8 cattle and 3 sheep. The most affected regions were the south-central and northwestern parts of the country.
- Based on our newly generated data, Hungary is now categorized as a level 3 (high potential) country considering the risk of CCHFV introduction and future human infections.

Although the seroprevalence determined both in the human population and among indicator animals can be considered low, especially compared to the seroprevalence data in certain hyper-endemic areas, it clearly indicates some degree of CCHFV presence in the country. As next step, we would like to assess the current seroprevalence in the country to gain important information regarding the spatio-temporal emergence of CCHFV since 2017. We also aim to perform active and comprehensive surveillance focusing on the principal tick vectors present and the CCHFV strains that potentially circulate in the affected geographical areas of Hungary. Moreover, our results highlight the importance of raising awareness among healthcare workers and other at-risk populations of the emerging threat of CCHFV in Hungary and Central Europe.

6. Bibliography of publications

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