COMENIUS UNIVERSITY IN BRATISLAVA

Faculty of Natural Sciences

UNIVERSITY OF CAGLIARI

Department of Biomedical Sciences

MOLECULAR EPIDEMIOLOGY OF HANTAVIRUSES IN CENTRAL EUROPE AND ANTIVIRAL SCREENING AGAINST ZOONOTIC VIRUSES CAUSING HEMORRHAGIC FEVERS

DISSERTATION

2017 RNDr. PaedDr. Róbert SZABÓ

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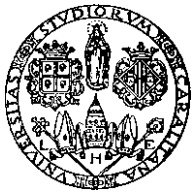

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Molekulárna epidemiológia hantavírusov v strednej Európe a antivírusový skríning proti zoonotickým vírusom spôsobujúcim hemoragické horúčky

Cieľ: Main objectives of the thesis include a molecular identification and phylogenetic analyses of the hantaviruses causing hemorrhagic fevers obtained from their reservoirs. Additional aim is the evaluation of the biological activity of selected newly synthesized aryl-piperazine derivates against the viruses causing hemorrhagic fevers belonging to the genera Orthohantavirus and Flavivirus. Secondary objectives involve a screening of the same series of aryl-piperazine derivate compounds against other representative viruses such as Vesicular stomatitis virus, Vaccinia virus, Coxsackie virus and HIV-1 which could provide an useful information of the importance of the polarity and/or segmentation of the viral genome for the antiviral activity of the inhibitors and also against different types of cancer cells.

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ABSTRAKT

Róbert Szabó: Molekulárna epidemiológia hantavírusov v strednej Európe a antivírusový skríning proti zoonotickým vírusom spôsobujúcim hemoragické horúčky

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Puumala orthohantavirus (PUUV) a *Dobrava-Belgrade orthohantavirus* (DOBV) sú medicínsky najvýznamnejšie hantavírusy v strednej a západnej Európe. V tejto práci sme analyzovali celkovo 578 drobných cicavcov (druhy *C. glareolus, A. agrarius,* a *A. flavicollis*) na prítomnosť hantavírusovej RNA. Čiastkové sekvencie PUUV genómových segmentov boli získané z 51 hrdziakov, DOBV z 1 ryšavky tmavopásej. Fylogenetické analýzy všetkých troch genómových segmentov ukázali, že novoobjavené PUUV kmene zo Šumavy, Česká republika, sú najviac príbuzné kmeňom zo susedného Bavorského lesa, známej oblasti epidémií hantavírusových infekcií. Slovenské PUUV kmene boli zoskupené so sekvenciami z Bavorského lesa iba v analýzach M segmentu, nie S segmentu. Tento topologický nesúlad napovedá reassortment segmentov alebo, keďže sme analyzovali iba čiastkové sekvencie, homologickú rekombináciu. V ďalšej časti práce sme vyhodnotili sadu nových derivátov aryl-piperazínu na ich antivírusovú aktivitu proti PUUV a *Seoul orthohantavírusu* (SEOV), pretože ešte nebolo publikované, že by sa takáto skupina chemických látok niekedy testovala proti hantavírusov a tiež preto, že predtým už vykazovali široké spectrum biologického účinku proti iným vírusom. Taktiež sme deriváty aryl-piperazínu testovali proti YFV, CVB-5, VV, VSV a HIV-1. Na základe článkov prezentujúcich protirakovinový účinok sem sa rozhodli, že tie s vyššou cytotoxickosťou otestujeme tiež proti rôznym typom nádorových bunkových línií. Niektoré deriváty aryl-piperazínov boli aktívne iba proti CVB-5 a dva z nich proti nádorovým bunkovým líniám.

Kľúčové slová: Vírusy hemoragickej horúčky, Fylogenetické analýzy, Hantavírus, Flavivírus, Deriváty aryl-piperazínov, Antivírusový skríning, Protirakovinový skríning.

ABSTRACT

Róbert Szabó: Molecular epidemiology of hantaviruses in Central Europe and antiviral screening against zoonotic viruses causing hemorrhagic fevers

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Puumala orthohantavirus (PUUV) and *Dobrava-Belgrade orthohantavirus* (DOBV), are the most medically important hantaviruses in Central and Western Europe. In this study, a total of 578 small mammals (species *C. glareolus, A. agrarius,* and *A. flavicollis*) from Germany, Slovakia, and Czech Republic trapped between the years 2007-2012 were analyzed for the presence of hantavirus RNA. Partial PUUV genome segment sequences were obtained from 51 voles, DOBV from 1 stripped field mouse. Phylogenetic analyses of all three genome segments showed that the newfound PUUV strains from Šumava, Czech Republic, are most closely related to the strains from the neighbouring Bavarian Forests, a known hantavirus disease outbreak region. The Slovak PUUV strains clustered with the sequences from Bohemian and Bavarian Forest only in the M but not S segment analyses. This topological incongruence suggests a segment reassortment event or, as we analyzed only partial sequences, homologous recombination. In the next part of the study, we evaluated a set of novel aryl-piperazine derivates for their antiviral activity against PUUV and *Seoul orthohantavirus* (SEOV), because it has been not yet published that this group of chemical compounds was ever evaluated against hantaviruses and they showed a great broad-spectrum biological activity against other viruses. We evaluated these aryl-piperazine derivates also against YFV, CVB-5, VV, VSV, and HIV-1. According to papers presenting anti-cancer activity, we decided to evaluate those with higher cytotoxicity also against different types of tumoral cell lines. Some of the aryl-piperazine derivates were active only against CVB-5 and two of them were active against tumoral-cell lines.

Key words: Hemorrhagic fever viruses, Phylogenetic analyses, Hantavirus, Flavivirus, Aryl-piperazine derivates, Antiviral screening, Anti-cancer screening.

PREFACE

Viral hemorrhagic fevers, particularly those virus species from the family *Flaviviridae* which are transmitted by mosquitoes are threatening people at least since early 18th century after their export to the New World from Africa when they caused several large outbreaks. World Health Organization (WHO) pays an attention to Hemorrhagic fever with renal syndrome (HFRS) since 1985, when scientists were able to identify hantaviruses as origins of hemorrhagic fevers not only in Asia, but in Europe, too. HFRS is known also as *Nephropathia epidemica* (NE). This name is used by authors describing HFRS caused by *Puumala orthohantavirus* in Fennoscandia (Scandinavia and Finland) since the disease was first described in this region by Swedish authors in 1934. Then, HFRS was recognized also in Western Europe, Central Europe, Balkans and Italy, as well. Milota Grešíková published the memoir *Hemorrhagic fever with renal syndrome.* Her work started the interest for a virological research of hemorrhagic fevers in Czechoslovakia (since 1993, Czech republic and Slovakia). Daniel Carleton Gajdusek, a co-recipient of Nobel Prize in Physiology or Medicine, whose parents came from Slovakia, also showed an increased interest for hemorrhagic fevers, especially those which are caused by hantaviruses.

This thesis is focused on basic research of hantaviruses received from their reservoir hosts from the regions of Central Europe. Updating the informations about hantavirus genome informations, their molecular evolution as well as the evolution of their reservoirs still remain an important approach in virological research due to the re-emergences of these viruses in last years and increasing number of infected persons annualy. It is not less important to pay attention also to other viruses causing hemorrhagic fevers, which significantly threaten the humankind. Even though these viruses are known for years, there is still a lack of antiviral therapeutics and prophylactics. Ribavirin, which was discovered in 1970, still remains the only one antiviral, available. According to these challenges, we decided to search a new antivirals which could have shown a sufficient activity against the virus replication cycle and which could have been used once also in clinical practice.

Róbert Szabó

CONTENTS

Introduction

Nowadays, viruses which can develop hemorrhagic fevers in humans are divided into the families *Nairoviridae, Peribunyaviridae, Hantaviridae, Phenuiviridae, Arenaviridae, Flaviviridae, Filoviridae, Paramyxoviridae,* and *Rhabdoviridae.* They differ by their pathogenic potential, way of transmission, host range, reservoir hosts, and geographical distribution. The hemorrhagic fever still remains as their common symptom.

Hantaviruses are known relatively for a short time in comparison with other viruses (a several decades). Their reservoirs are small mammals from the orders of rodents, insectivores, and as it has been recently shown, bats, too. The most challenging viruses causing hemorrhagic fevers among flaviviruses are Yellow fever virus, Dengue virus, and West Nile virus. In contrary to hantaviruses, these flaviviruses are transmitted by mosquitoes. All the mentioned groups of viruses represent a serious challenge in public health due to the lack of antivirals and a sufficient prophylaxis with exception of Hantavax, the only available vaccine against hantaviruses only in East Asia. Vaccine against Yellow fever virus came into use in 1938. It provides a long-life immunity just after a single dose. Dengue virus is another case which is still problematic and the experiments of developing a vaccine protecting humans are mostly unsuccessful as well as with West Nile virus.

Even though the term Neglected Tropical Diseases was mentioned for couple of years, at least since the Ebolavirus outbreaks in 2014 it is obvious, that this term belongs to the past and viral infectious diseases challenge us to develop new and effective antivirals and vaccines to reduce not only numerous human life casualties, but also to improve a general welfare. It is assumed that global changes responsible for such a large outbreak of Ebolavirus have also a responsibility for increasing number of infections also by other hemorrhagic fever viruses, such as flaviviruses and hantaviruses, annually. Thus, it is important to pay attention to development of new antivirals as well as effective vaccines.

1. BACKGROUND

1.1 Global burden of infectious diseases and hemorrhagic fevers

Estimated number of annual human casualties which are directly caused by infectious diseases in general is about 17 million, out of 57 million annual deaths worldwide. Approximately 60% of recently emerging infectious diseases infecting humans have a zoonotic origin and 70% have the origin in wild nature (Jones *et al.*, 2008). The outbreak of the Ebola virus between 2014 - 2016 in West Africa (Baize *et al.*, 2014) shows that global changes in last decades help to spread viruses more effectively. Since the discovery of the deadly Ebola virus in 1976, several previously unknown pathogenic viruses have been discovered. Besides of the Ebola virus, there are other clinically important taxonomic groups of viruses causing hemorrhagic fevers. The whole family *Hantaviridae* is particularly known in association with hemorrhagic fevers. Among the family *Flaviviridae*, the genus *Flavivirus* is that one where the viruses are known as the origins of hemorrhagic fevers in humans. Due to the hemorrhagic manifestations, these viruses are also referred as hemorrhagic fever viruses. A lot of species of flaviviruses are called as arboviruses for their transmission by arthropode vectors (ticks or mosquitoes) (Lin *et al.*, 2003; Klempa *et al.*, 2013).

Aside to these emerging infectious diseases (EIDs) stands the increase of the "re-emerging" infectious diseases, rising over the last decades the public concern. While emerging infections have never been previously recognized in men, re-emerging infections, detected and described in the past, are rapidly increasing in the present. The range of the infections can be measured by the number of incidence, by the host range and by the geographical distribution. Emerging diseases are generally determined by these parameters of infection: (viral) evolution and adaption, human host, animal reservoirs, ecology (Ghosh and Dar, 2015).

Some of the emerging and re-emerging infections are also classified as "Neglected Tropical Diseases" (NTDs). NTDs with hemorrhagic fever viruses included, threaten over 1 billion people, mainly in extremely poor areas of Africa, Asia and Americas (Jones *et al.*, 2008; Morens *et al.*, 2008; web source 1). Besides approximately 20 viruses, funghi end ectoparasites, NTDs involve at least 13 parasitic

(helmintic and protozoal) and bacterial infections. Due to the relatively low mortality and the endemic occurrence, mostly in slums of developing countries, NTDs are generally considered as low priority diseases. If NTDs are defined as a group of tropical infectious diseases in developing countries, which are both poverty-promoting and long-lasting in their public health impact, then arboviruses (arthropod-borne viruses) certainly belong to NTDs. Arboviruses are taxonomically divided into independent groups representing at least 8 families and 14 genera. There are 534 viruses registered in the International Catalog of Arborviruses. 134 of them are known as origins of human diseases and approximately 40 of them are possible to infect a livestock and develop a disease in them. The most clinically important arboviruses causing human diseases belong to the families *Flaviviridae,* and *Togaviridae* (Lozano *et al.*, 2012; Raso *et al.*, 2004; Nalca *et al.*, 2003; Rust, 2012).

Major part of viral zoonoses, and particularly those ones which involve wild animals and arthropodes seem to be impossible to eradicate. Over the last decades, their evolution has been rising and expanding as a consequence of several factors (i.e. export and/or expansion of suspectible host populations, crossing of species' barrier, effect of global warming on vectors' distribution and spread), which facilitate and increase the contact rate between humans and source of infectiousness (Cutler *et al.*, 2010).

1.2 Historical overview of viral hemorrhagic fevers

Viral hemorrhagic fevers (VHFs) burden the mankind for thousands of years, but it took a time when people began to be able to characterize diseases and identify their origins. The oldest reported evidence of hantavirus disease, hemorrhagic fever with renal syndrome is preserved from the 10th century in China during the reign of five dynasties and ten kingdoms (五代十国) (Lee *et al.*, 1982). More sofisticated reports appeared later by the beggining of 20th in Siberia (Casals *et al.*, 1970). In the period of Korean war (1950 - 1953), around 3,000 soldiers got sick. The disease was called Korean hemorrhagic fever (KHF), nowadays known mainly as Hemorrhagic fever with renal syndrome (HFRS) (Grešíková *et al.*, 1988). In Europe, HFRS is known since 1934. First reports came from Scandinavia (Lähdevirta, 1971; Brummer-Korvenkontio *et al.*, 1980). In New World, hantaviruses are known since 1993 when extremely lethal *Sin Nombre orthohantavirus* infected a couple of men in the area of Four Corners and the illness was named as Hantavirus (cardio-) pulmonary syndrome (HPS, HCPS) (Nichol *et al.*, 1993).

The origins of *Yellow fever virus* (YFV) are most likely from Africa (Gould *et al.*, 2003). Based on phylogenetic analyses, the virus was spread from East or Central Africa to the west by humans and other primates (Bryant *et al.*, 2007). YFV is circulating in Americas probably since the end of 15th century when the infected vector of this virus, *Aedes aegypti*, a mosquito species, was probably brought by ships within the transatlantic slave trade (Haddow, 1969). The first reported outbreaks of disease caused probably by YFV were on Barbados in 1647 and Guadalupe in 1648 (McNeil, 2004). Then, at least 25 huge outbreaks occured in North America, such as in 1793 in Philadelphia, where more than 5,000 people died. Then, during the 19th century several outbreaks occured in south of the USA in Georgia, Louisiana, Virginia, Texas and Louisiana, and Lower Mississippi Valley in the years 1820, 1853, 1855, 1867 and 1878, respectively. The last big outbreak of YFV in the US was in 1905 in New Orleans (Kohnke, 1906). By the time, three outbreaks of YFV occured in Barcelona, Spain in 1803, 1821 and 1870. In the last outbreak, 1,235 victims died of the total of 12,000 infected. Barcelona was one of the major arrival ports for ships from the Carribean from where was the virus imported (Canela Soler *et al.*, 2009). In Africa, YFV did not

disappear. In Ethiopia, between 1960-62 were 30,000 deaths of the total of 200,000 cases (Haddow, 1969). For the year 2013, it was estimated 130,000 cases of yellow fever in Africa with fever and jaundice or hemorrhage including 78,000 deaths, taking into account the current level of vaccination coverage (Garske *et al.*, 2014).

West Nile virus (WNV) was isolated for the first time from the West Nile district of Northern Uganda in 1937 (Smithburn *et al.*, 1940). The epidemiological potential of WNV and its ecology was first described and characterized after several outbreaks in the Mediterranean basin in 1950s and 1960s (Sejvar, 2003). In the New World, WNV was firstly detected in 1999, when a group of severe cases of encephalitis was reported in the area of Queens in New York City (Nash *et al.*, 2001). The virus was spread through birds and it infected mammals through mosquitoes. Since 1999 through 2015, the estimated number of WNV cases varied from 2.6 to 6.1 million people in the USA. In more than 41,000, the virus developed a severe illness from which more than 1,700 died (Grinev *et al.*, 2017). Large outbreaks of WNV, particularly among equines, were detected in Italy in 1998 and 1999 (Cantile *et al.*, 2000) and in France in 2000 (Murgue *et al.*, 2001).

Dengue virus (DENV) outbreaks were clinically reported for centuries, but the origin of the disease was not characterized until the first virus isolations in 1943 in Japan and in 1945 in Hawaii (Messina *et al.*, 2014). For now, DENV circulates in at least 100 countries in Asia, the Pacific, the Americas, Africa, and the Carribean. According to WHO estimates, from 50 to 100 milion of cases and 22,000 deaths are reported annualy (Bhatt *et al.*, 2013).

Even though our work is focused mainly on viruses from the families *Hantaviridae* and *Flaviviridae*, it is worth to mention some historical highlights of Lassa virus (family *Arenaviridae*) and Ebolavirus (family *Filoviridae*), which are the origins of severe and highly lethal hemorrhagic fevers in Africa. *Lassa mammarenavirus* is known since 1969 when caused a couple of outbreaks of various magnitude and severity in West Africa. For now, estimated number of annual cases of infections in this area is between 300 - 500,000 and around 5,000 are fatal (Ogbu *et al.*, 2007). Since 1969 until 2004 several of Lassa fever patients brought the virus from Nigeria and Sierra Leone to the USA, Canada, UK, Germany, Netherlands, Israel and Japan (Macher and Wolfe, 2006).

Ebolavirus is known since 1976 after the outbreak in Sudan and Zaire (WHO, 1978 a,b). Major outbreaks then occured in Democratic Republic of Congo (formerly Zaire) in 1995 (Khan *et al.*, 1999), in Uganda between 2000-2001 (Okware *et al.*, 2002), and in Democratic Republic of Congo again in 2007 (WHO, 2007). For each of these outbreaks, the number did not reach over 425 of cases and 280 of deaths. On the other hands, during the huge outbreak between 2014 - 16 Ebolavirus infected more than 28,000 people from which at least 11,000 died. The infection attacked mutliple countries in West Africa and the virus was also transported to the USA, Spain, Italy and United Kingdom (Lamunu *et al.*, 2017; Omoleke *et al.*, 2016; Sueblinvong *et al.*, 2015; Cerón-Serrano *et al*., 2016; Bertoli *et al.*, 2016; Jacobs *et al.*, 2016).

1.3 Virus nomenclature, and classification

1.3.1 Family *Hantaviridae*

The genus *Orthohantavirus* representes for now the only genus within the family *Hantaviridae* (order *Bunyavirales*). It involves 41 species according to International Comittee on Taxonomy of Viruses (ICTV) from 2016 (web source 2). Hantaviruses are not classified as arboviruses ("Arthropode Borne Virus"), so they are transmitted directly through the air by aerosilized excreta (urine, saliva and feces) of their reservoir hosts (rodents, shrews and bats). At the beginning, it had been believed, that reservoir hosts of hantaviruses are rodents only (order *Rodentia*), but it has been demonstrated later, that the reservoir host range includes small mammals from the groups of insectivores (order *Insectivora*) and bats (order *Chiroptera*), as well (de Oliveira *et al.*, 2014).

The nomenclature of hantavirus species is usually based on the name of geographic locality where they were found and isolated for the first time. The hantavirus species demarcation criteria are defined by the ICTV. Until 2016, there had been four rules for the demarcation of independent species among hantaviruses. Individual hantavirus species should had been found in unique ecological niches, i.e. in different primary rodent/insectivore reservoir species. According to the second rule, the hantavirus species had exhibited at least 7% difference in aa identity on comparison of the complete glycoprotein precursor and nucleocapsid protein sequences (there were some exceptions presumably caused by historically recent host-switching events). The third rule had said, that species should had showed at least four-fold difference in two-way cross neutralization tests. And according to the fourth rule, the species had done not naturally form reassortants with other species (Plyusnin *et al.*, 2012).

Then, the hantavirus genome was analysed more complexly and it was believed, that some rules of ICTV could be misleading. So, it was suggested, that individual hantavirus species should have shown at least 10% difference in S segment and 12% difference in M segment of complete aa sequences (Maes *et al.*, 2009).

In 2016, the ICTV *Bunyaviridae* Study Group agreed to state new criteria of hantavirus species demarcation, which are based only on the genome sequences. Basically, to demarcation an independent virus species, it is necessary to:

- make an amino acid concatenated multiple alignment containing the coding-complete regions of the nucleocapsid protein (S segment) and glycoproteins (M segment);
- calculate PED values using WAG amino acid substitution matrix (Tree-Puzzle, maximum likelihood parameter);
- a species of the genus *Hantavirus* (proposed *Orthohantavirus*) is defined by a PED value for its member greater than 0.1.

For now, all 24 proposed species meet the criteria to be demarcated as distinct *Hantavirus* (proposed *Orthohantavirus*) species of a 0.1 PED threshold for each member virus (Figure 1). Full list of newly classified hantavirus species is available online (web source 2).

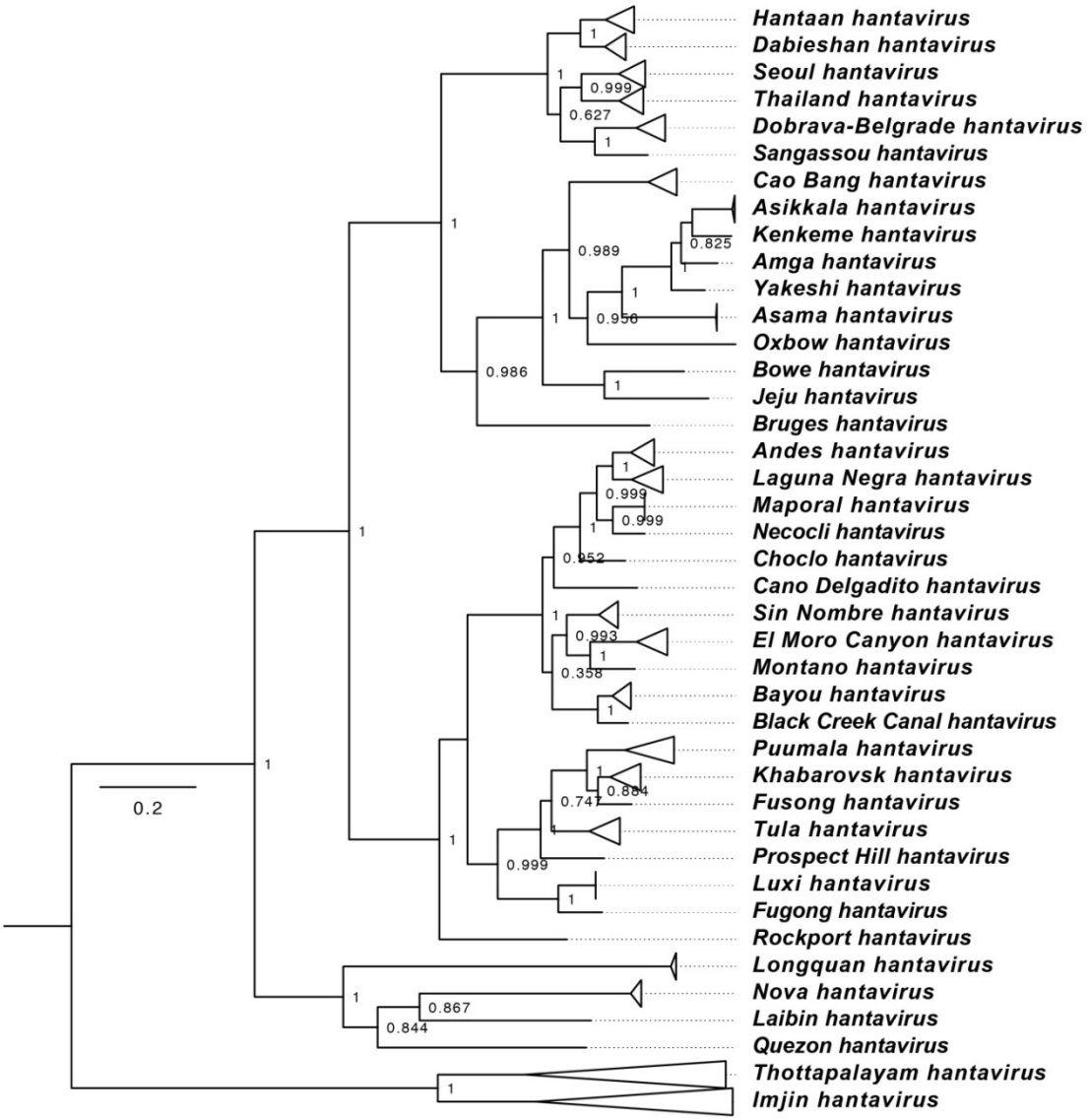

Figure 1: Bayesian MCMC phylogenetic tree of a concatened S+M full coding region (amino acids) dataset. Internal nodes of the tree that correspond to *Hantavirus* species are collapsed (triangles); all of them have bootstrap support values of 1 (100%). Note that all species name in this table would be changed to the format "*Xxx orthohantavirus*" if this taxonomic proposal is accepted in its entirety (web source 2).

1.3.2 Family *Flaviviridae*

Major part of the viruses from the family *Flaviviridae* are primarily spread through arthropod vectors (ticks and mosquitoes), so they are considered as "arboviruses". There are currently known over 100 species of the viruses in this group divided into four genera: *Hepacivirus*, *Pegivirus*, *Pestivirus*, and *Flavivirus* (web source (web source 3).

Hepacivirus C (HCV) (genus: *Hepacivirus*) also known as Hepatitis C virus is the most medically important virus among hepaciviruses. The disease is also called Hepatitis C and the virus was discovered in 1989 (Choo *et al.*, 1989; Kuo *et al.*, 1989). HCV is transmitted by blood-to-blood contact due to unprotected intravenous drug use, and infected transfusions. Another possible way of the virus transmission is from an infected mother to baby during the birth. Although rare, HCV can be transmitted through sexual intercourse, as well (Terrault *et al.*, 2013). Untreated infection is a leading cause of cirrhosis and hepatocellular carcinoma which is the most frequent of liver failure requiring transplantation. HCV is responsible for more deaths annually than human immunodeficiency virus (HIV). HCV infection may be asymptomatic for a time and approximately half of persons do not know about their infection. Within 30 years, at least 41% of HCV-positive patients progress to cirrhosis, leading to liver failure, hepatocellular carcinoma, and death from liver-related causes (Lingala and Ghany, 2015). It is estimated, that 2% of the global population (71 million people) have chronic HCV infection. Approximately 3-4 million people are infected per year and 400,000 die from cirrhosis and/or hepatocellular carcinoma. Current antivirals can cure more than 95% of persons with HCV infection, but there is still no vaccine avialable (web source 4).

The genus *Pegivirus* was classified in 2011 (Stapleton *et al.*, 2011). This group includes 11 species: Pegivirus A-K infecting human, horses, pigs, rodents, and bats (Smith *et al.*, 2016). Human pegivirus (HPgV; known also as GB virus or hepatitis G virus) disposes by a limited pathogenicity. HPgV is mostly associated with prolonged survivals in HIV-infected patients. The virus was thought to be hepatotropic. Early studies found HPgV RNA in liver biopses, but the virus was detected in spleen, bone marrow, cerebrospinal fluid, PBMCs, and T- and B-lymphocytes, too (Chivero *et al.*,

2014). It has been reported, that according to study from southern Brazil where HPgV has been circulating, that HIV/HPgV coinfection was associated with a longer survival among HIV-positive patients as well as the coinfection by HPgV is more suspectible for the patients infected by HIV non-C subtypes (Da Mota *et al.*, 2016).

Virus species within the genus *Pestivirus* are reported to involve diseases followed by bleeding disorders, abortions, diarrhoea, fatal mucosal disease, and respiratory difficulties in mammals such as cattle, sheep, goats, and swine. Among the wild animals, they infect giraffes in african savannah and pronghorns in american prairie. In Australia, they are widespread, mainly in cattle. Some adult cattle can develop the imunity to the disease. Pestiviruses have been not reported to be harmful to humans. The most known species are Bovine viral diarrhoea virus (BVDV), Border disease virus (BDV), and Classical swine fever virus (CSFW) (Giangaspero and Harasawa, 2011).

In a study, a foetal calf sera (FCS) used for laboratory properties in cell cultures were tested for the suspicion of the pestivirus presence. BVDV RNA was detected in 33% of the vaccine lots using the FCS. The BVDV RNA detected in human live viral vaccines represents passive carry over of BVDV from contaminated FCS rather than active virus replication in human diploid cells. The results of the study reporting the contamination of FCS by BVDV used in vaccines indicate that the BVDV RNA presence is not a reason of serious concern to human health. Moreover, it has been demonstrated, that the gamma-irradiation of FCS destroys BVDV particles and can be also effective in prevention of BVDV RNA in the vaccines (Studer *et al.*, 2002).

The most known species within the genus *Flavivirus* are *West Nile virus* (WNV), *Dengue virus* (DENV), *Yellow fever virus* (YFV), Zika virus, *Tick-borne encephalitis virus* (TBEV). First three of them are mosquito-borne viruses mostly known for the hemorrhagic manifestations in humans. TBEV is strictly transmitted by ticks and causes tick-borne encephalitis which can result in permanent brain damage (Kaiser, 2008).

Zika virus started to concern people since it appearance in early 2015 in Brazil. Then, the outbreaks spread to other parts of South and North America. It affected also several islands in the Pacific, and Southeast Asia. Microcephaly caused by Zika virus represents the epilogue of infection's devastating process on the central nervous system of embryos and fetuses. Zika virus can cause also arthrogryposis, dysphagia, deafness,

and visual impairment. Another reason of concerns is the fact, that the virus had spread itself in such a range of the Americas, even though its origin is in Africa (Duarte *et al.*, 2017; Metsky *et al.*, 2017; web source 5).

The species demarcation criteria within the genus *Flavivirus* include 7 parameters: Nucleotide and deduced amino acid sequence data; Antigenic characteristics; Geographic association; Vector Association; Host association; Disease association; and Ecological characteristics. All the species within the genus are classified according each of the mentioned criteria. Nucleotide sequence is considered as the most important information for the species demarcation. Other criteria may serve a useful information among genetically closely related species of the viruses. In a case of tick-borne encephalitis virus, far-eastern (FE) strains exhibit distinct ecological differences in comparison with Omsk hemorrhagic fever virus (OHFV), even though they are genetically closely related. TBEV-FE is predominantly carried by ticks from the genus *Ixodes*, whereas OHFV is more associated with ticks from the genus *Dermacentor*. The antigenic differences in neutralization tests were demonstrated, as well (Simmonds *et al.*, 2012; web source 3).

Another case is a comparison of *Louping ill virus* (LIV) with TBEV which showed both, a close genetic likeness and a similar host range. Despite of that, they dispose by different ecologies (moorlands verus forests), pathogenicities (red grouse, sheep/goats versus humans) and geographical distributions (United Kingdom versus Europe/Asia). According to the mentioned parameters, they have been classified as different species.

Dengue virus (DENV) is an example of a different kind. The four serotypes of DENV are altogether classified within the same virus species, despite of that they are phylogenetically as well as antigenically quite distinct. The species determination was based on the same geographical area where they circulate. All the DENV serotypes are harbored by the identical species of vectors. Their virus replication cycle is very similar and they cause similar clinical manifestations, too (Simmonds *et al.*, 2012; web source 3).

1.4 Virus genome structure, and replication

1.4.1 Genus: *Orthohantavirus*

The genomic structure of hantaviruses (genus *Orthohantavirus*) is common for the whole family *Hantaviridae.* The virions are enveloped, pleomorphic particles and contain negative-sense single-stranded RNA. The tripartite genome contains a small (S)-segment, medium (M) segment, and large (L) segment encoding a nucleocapsid (N) protein, a glycoprotein precursor (GPC), and the viral RNA-dependent RNA polymerase (RdRp), respectively (Figure 2). Each segment is directly associated with molecules of RdRp (Schmaljohn and Nichol, 2007). Terminal parts of each segment are non-covalently connected by hydrogen bonds and create "panhandle" structures, which are one of the characteristics of the order *Bunyavirales* (Mir and Panganiban, 2005).

N protein is the most numerous protein present in a matured virus particle encapsidating each of the hantavirus genome segments and can be detected early 6 hours post infection (hpi) (Spiropoulou, 2001). N protein plays a role of protection protein of the vRNA (virus RNA) against the nuclease degradation during the encapsidation of virus particle. N protein as a part of the ribonucleoprotein (RNP) is directly connected to the envelope by a cytoplasmatic tail on the G1 protein and plays a role in the initiation of virus maturation. That is most likely the reason why hantaviruses do not have a necessity of having a matrix protein. The absence of a matrix protein is one of the characteristics of the whole order *Bunyavirales.* N protein is also important in prevention of intranuclear base pairing within the vRNA template (Kolakofsky and Hacker, 1991; Petterson, 1991). S segment contains an additional putative ORF coding non-structural protein (NSs) of 120-270 nt. Interestingly, NSs is produced only by hantavirus species associated with the rodents of the subfamilies *Arvicolinae* and *Sigmodontinae*. On the contrary, NSs absents in *Murinae*-associated hantaviruses. It has been demonstrated, that the NSs protein of PUUV and Tula virus is potent to inhibit the activation of interferon-beta promoter (Jääskeläinen *et al.*, 2007; Jääskeläinen *et al.*, 2008).

Hantavirus glycoprotein precursor (GPC) is synthesized on the ribosomes of rough endoplasmic reticulum (ER). Next, GPC is spliced on the membranes of ER to

the G1 (Gn) and G2 (Gc), which create the hantavirus envelope. It has been demonstrated, that G1 and G2 are dependent to each other. Individually expressed G1 and G2 persist in ER and are not able to transport themselves onto the membranes of the Golgi apparatus, from where the matured virus particles are commonly released (Pensiero and Hay, 1992; Deyde *et al.*, 2005; Khaiboulina *et al.*, 2005).

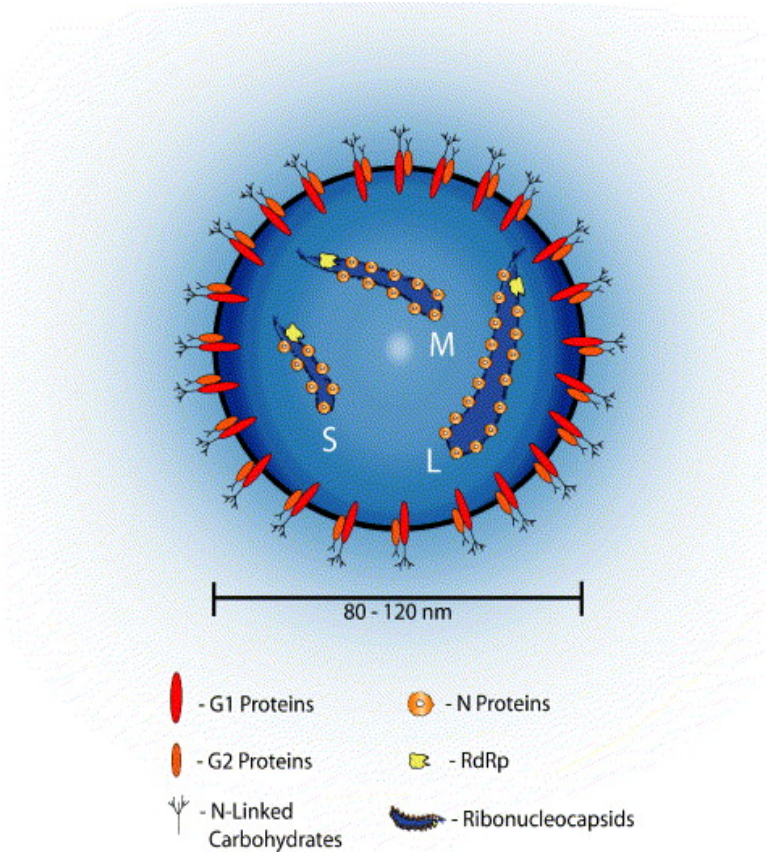

Figure 2: The structure of the hantavirus virion (Jonsson *et al.*, 2005).

It has been shown, that the pathogenic hantavirus species attach cellular β3 integrin receptors for the cell attachment (Guerrero *et al.*, 2000; Gavrilovskaya *et al.*, 2008). Non-pathogenic or low-pathogenic hantaviruses enter the cells through β1 integrins (Gavrilovskaya *et al.*, 2002). The coreceptor DAF/CD55 (decay-accelerating factor) anchored on the GP1 protein also plays its role in virus adsorption. The infection of *Puumala orthohantavirus* was inhibited by the application of DAF-specific antibodies on the epithelial and endothelial cells (Krautkrämer and Zeier, 2008).

The whole hantavirus replication is situated in the cytoplasm. RdRp initiates the synthesis of the positive mRNA from the genomic RNA (of S, M and L segments) by

“cap-snatching” mechanism. Hantaviruses absent the cap structure, so they take it from the host cell to initiate the transcription (Dunn *et al.*, 1995). Initiation of transcription seems to be similar to that of influenza viruses, in which the endonuclease associated with the polymerase complex cleaves the host cells’ mRNAs to generate capped fragments (7-18 nt) which act as primers, and the presence of a methylated 5’ cap structure on the host mRNA is required for occurrence of this cleavage of influenza viruses as well as of hantaviruses (Garcin *et al.*, 1995).

The hantavirus L protein is associated with the RNPs and it initiates viral RNA synthesis immediately after the virus entry into a host cell. L protein cleaves 7-18 nt long primers for virus mRNA synthesis from the 5′ termini of cellular mRNA molecules present in the cytoplasm. The transcription continues by the mechanism “prime-and-realign” (or slip-back or jump back), when the copy of the 3′ end vRNA is synthesized. At the 3′ end is a G nucleotide which pairs with the C nucleotide at the position 3 on the viral RNA tempalate (3′AU**C**AUCAUC...). When the primer is shortly elongated, the newly synthesized RNA shifts three nucleotides backwards and realigns with the template. Interestingly, the transcribed viral mRNA is shorter than the original viral RNA template and its exact complementary copy. According to the antigenomic cRNA, the transcription is termined just before the RdRp reaches the 5′end of the RNA template. The exact mechanism how the mRNA synthesis is completely done and terminated is still largely unknown (Hutchinson *et al.*, 1996; Kaukinen *et al.*, 2005). The studies of the mRNA synthesis showed that 3′ end of the L segment mRNA is not polyadenylated as well as in the case of S segment mRNA. Only the 3′ end of the M segment mRNA was polyadenylated. The termination of synthesis of M mRNA initiates on the U8 polyadenylation-transcription signal, which is considered as a highly conserved among hantaviruses (Abraham and Pattnaik, 1983; Hutchinson *et al.*, 1996).

1.4.2 Genus: *Flavivirus*

The genome structure of the viruses from the genus *Flavivirus* is very similar. A single-stranded positive-sense RNA genome coding a single polyprotein is more than 10 kb long (a single ORF). The genome is flanked by NCRs at the 5' and 3' ends. Both, the 5' and 3' NCRs dispose by specific motifs which have been demonstrated to be involved in viral RNA translation, replication and probably packaging, too. The viral RNA naturally contains some structural and functional parts which are conserved and characteristic for all the species within the genus *Flavivirus*. On the other side, some genome characteristics such as sequence order and genome length can vary from species to species within the same genus. Moreover, it can vary among the serotypes within the same species. 3' NCR of some TBEV isolates contained an internal poly(A) tail (Chambers *et al.*, 1990).

Flaviviruses generally cause a dramatic rearrangement of the cellular endoplasmatic reticulum (ER) membrane and lead to formations of ER-derived packets where the viral replication cycle goes on. After translation of the entered vRNA, RNA replication starts by synthesising the complementary negative strands, which next serve as templates to produce genome positive-stranded molecules. These are synthesized by a semi-conservative mechanism with replicative intermediates (double stranded regions and nascent single stranded molecules) and replicative molecules (duplex RNA). The translation starts classically at first the AUG of the ORF, but sometimes may start at a second in-frame AUG at 12 – 14 codons downstream in mosquito-borne flaviviruses (Simmonds *et al.*, 2012; web source 3).

Then, the polyprotein is next post- and co-translationally spliced into three structural proteins- C the major envelope (E) protein, and either prM (in immatured virions), or M (in matured virions), and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) by the mechanisms involving cellular proteases and the viral NS2B-NS3 serine protease. The envelope (E) protein is the largest among structural proteins. It is the main component of the virion surface (Figure 3). Besides that the E protein is the primary immunogen, it plays its role in receptor binding and membrane fusion. Virus particles can be first visibly present in the rough endoplasmatic reticulum, which is most probably the site of the virion assembling. Immature virus

particles are then transported to the cell membrane where escape the cell by exocytosis. Before the release of the virion off the cell surface, the viral prM protein is cleaved by furin or a furin-like cellular protease (Heinz and Allison, 2003). Besides the viruses, the flavivirus-infected cells are releasing a non-infectious subviral particles which have a lower coefficient of sedimentation than the whole virus (70S : 200S). These subviral particles exhibit hemagglutination activity (Simmonds *et al.*, 2012; web source 3).

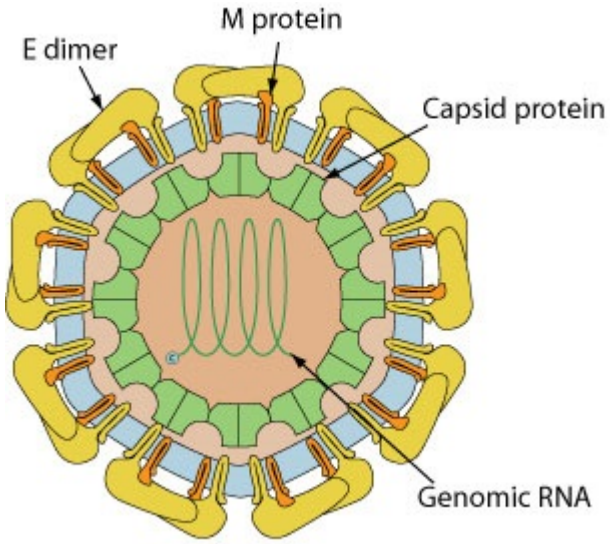

Figure 3: Diagram of a Flaviviridae virion. The membrane is composed of three types of peoteins (E, M, C) (web source 6).

1.5 Virus ecology, transmission and epidemiology

1.5.1 Genus *Orthohantavirus*

Hantaviruses have been for decades considered as only rodent-borne viruses. Then, it was revealed, that the reservoir host range of hantaviruses includes insectivores (order: *Insectivora*) and bats (order: *Chiroptera*), too. In 2007, Tanganya virus was discovered as the first known hantavirus transmitted by insectivore (Klempa *et al.*, 2007). In fact, first discovered was Thottapalayam virus, but it was considered as an exception among hantaviruses for a long time (Song *et al.*, 2007a). A couple of months later, another two insectiovore-associated hantaviruses were discovered: Camp Ripley virus (RPLV) hosted by *B. bevicauda* from Minnesota (USA) (Arai *et al.*, 2007) and Cao Bang virus (CBNV) hosted by *A. squamipes* in China. In Europe, the first known insectivore-borne hantavirus was Seewis virus (SWSV) hosted by *S. araneus* (Song *et al.*, 2007b,c).

The bats, as a newfound reservoir host of hantaviruses play their particular role in virus ecology due to the fact, they represent an animal host moving by air in numerous groups what could increase the infectiousness and the spread of virus. Bioinformatic analyses of Magboi virus (MGBV) hosted by *N. hispida*, the bat species from Sierra Leone showed the highest molecular genomic similarity with hantaviruses associated with shrews and moles (Thottapalayan virus 73%, Altai virus 69.7%, Nova virus and Imjin virus 69.3% (Weiss *et al.*, 2012). Another discovery of bat-borne hantavirus was the Mouyassué virus (MOUV) hosted *N. nanus* from Ivory Coast in 2011 (Sumibcay *et al.*, 2012). In Gabon, Central Africa was then discovered Makokou virus (MAKV) hosted by Noack's Roundleaf Bat (*H. ruber*). In Europe, Brno virus (BRNV) was discovered in european bat species *Nyctalus noctula* (Straková e*t al.*, 2017). The pathogenic and epidemiological potential of bat-borne hantaviruses is not yet fully discovered.

Hantaviruses are hosted by their reservoir hosts and spread directly through the air by their aerosolized excreta. Some studies indicate, that infection via bites of wild animal is also possible, but it is less common (Schultze *et al.*, 2002). Humans are considered as "dead-end-hosts". It means, that the infection does not spread from human

to human. Human to human infection was confirmed only in several cases of Andes virus (ANDV), the endemic highly virulent hantavirus (within the *Andes orthohantavirus* species) circulating in South America. The major part of human to human infection cases were among the close relatives living in the same household or among the patients and a hospital staff which had been taking care of them. The efficiency of human-to-human transmission seems to be very low and requires very close contact. Therefore, the huge pandemics as in cases of some Influenza virus strains are not expected (Martinez *et al.*, 2005; Ferrés *et al.*, 2007).

The absence of the vector in replication cycle of hantaviruses seems to be crucial in their evolution which is dependent on the evolution of their reservoir hosts. For most of the last 50 years rodents have been considered to be the primary hosts of hantaviruses. The theory of coevolution of hantaviruses and their rodent hosts was supported by the results of phylogenetic analyses which revealed three evolutionary lines of hantaviruses parallel with phylogeny of three families of rodents (*Murinae, Arvicolinae, Sigmodontinae*) based on their cytochrome b nucleotide sequences (Hughes and Friedman, 2000). Hantaviruses associated with animals from the order *Soricomorpha* create an independent evolutionary line (Charrel *et al*, 2011). The closest common ancestor of these animal hosts appeared probably 50 million years ago. (Hoffmann and Koeppl, 1985).

"Host-switch" or/and "spill-over" were considered as only rare random events in hantavirus evolution (Vapalahti *et al.*, 1999; Klempa *et al.*, 2003; Plyusnin and Morzunov, 2001; Schmidt-Chasanit *et al.*, 2010). However, recent discoveries have revealed that hantaviruses infect a more diverse range of mammalian hosts, particularly *Chiroptera* (bats) and *Soricomorpha* (moles and shrews), and that cross-species transmission at multiple scales has played more important role in hantavirus evolution than assumed previously. Phylogenetic analyses suggest that hantaviruses might have first appeared in bats, shrews and moles before emerging in rodent species (Guo *et al.*, 2013; Holmes and Zhang, 2015).

1.5.2 Genus *Flavivirus*

1.5.2.1 Yellow fever virus

Transmission of the viruses causing hemorrhagic fevers belonging to the genus *Flavivirus* is closely associated with a vector, mainly mosquitoes. *Yellow fever virus* (YFV) is phylogentically closely related to the other viruses from the same family as *West Nile virus* (WNV), *St. Louis encephalitis virus* and *Japanese encephalitis virus* (Figure 4). YFV is transmitted primarily thorugh the bites of infected mosquitoes most commonly from the genus *Aedes* and *Haemogogus*. Mosquitoes get the virus by feeding on infected primates (human or non-human) and then transmit the virus to the other individuals. Mosquitoes can acquire the virus also from people which are considered as "viremic", which means a short time before the onset of fever and up to 5 days after onset of symptoms. The YFV transmission includes three types of cycles: jungle (sylvatic), intermediate (savannah), and urban. The jungle (sylvatic) transmission involves the circulation of the virus among mosquitoes and non-human primates in the forest. The virus is transmitted to the tourists or the men who are working in the jungle. An intermediate cycle present in African savannah represent a transmission of the virus from mosquitoes to the men living in jungle border areas. In the case of urban cycle, the virus is brought to the urban environment by an infected man who came from the jungle and savannah (web source 7).

For the urban cycle, the most important vectors of YFV are *A. aegypti*, *A. albopictus*. *A. aegypti* is the leading vector of YFV in South Asia. *A. africanus* seems to be a vector spreading the virus between the cities and rural areas of savannah in Africa. In South America, two main mosquito species spreading YFV are the genera *Haemagogus* and *Sabethes* (Galindo and Trapido, 1956; de Rodaniche and Galindo, 1957).

1.5.2.2 West Nile virus

The host range of *West Nile virus* (WNV) is different. WNV naturally circulates between mosquitoes (most commonly from the genus *Culex*) and birds. Some birds can

develop high levels of the virus in bloodstream and other mosquitoes become infected by biting them. Between 1999 - 2012 in USA, a total of 342 different bird species were analyzed from the dead specimens in which WNV was detected (web source 8). When mosquitoes acquire the virus, they can pass it to other birds biting them about a week after since they obtained the virus. The host range of WNV includes also people, horses and other species of mammals, which represent a "dead end" host, from whom the virus does not transfer further. Dead end hosts do not develop high virus levels for an effective pass of the virus to the other biting mosquitoes. Some groups of mammals (rodents, rabbits, and squirrels) and also reptiles (alligators) were found to develop a sufficient level of viremia to predict at least a low possibility for infecting other biting mosquitoes, but anyway, these cases are still very rare (Bowen and Nemeth, 2007).

Naturally, WNV is most commonly transmitted also to humans by mosquitoes. Additional routes of human infections have also been documented. It is important to note, that these ways of transmission represent a very small proportion of cases: Blood transfusions; Organ transplants**;** Exposure in a laboratory setting**;** From mother to baby during pregnancy, delivery, or breastfeeding.

It is also important to mention, that WNV is not transmitted (web source 9):

- From person to person or from animal to person through casual contact. Normal veterinary infection control precautions should be followed when taking care of a horse suspected to have this or any viral infection.
- From handling live or dead infected birds. Anyone should avoid bare-handed contact when handling any dead animal. If anyone is disposing of a dead bird, should use gloves or double plastic bags to place the carcass in a garbage can.
- Through consuming infected birds or animals. In keeping with overall public health practice, and due to the risk of known food-borne pathogens, anyone should always follow procedures for well cooking meat from either birds or mammals.

1.5.2.3 Dengue virus

Dengue virus (DENV) is harbored by mosquitoes, as well. It has been shown that *A. aegypti* is the main mosquito host of DENV. Other species of mosquitoes even if they are able to host DENV, they have limited ability to serve as DENV vectors and

acquire a sufficient level of the virus to spread the infection (Rodhain and Rosen, 2001). All the known possible vectors of DENV are mentioned in Table 1.

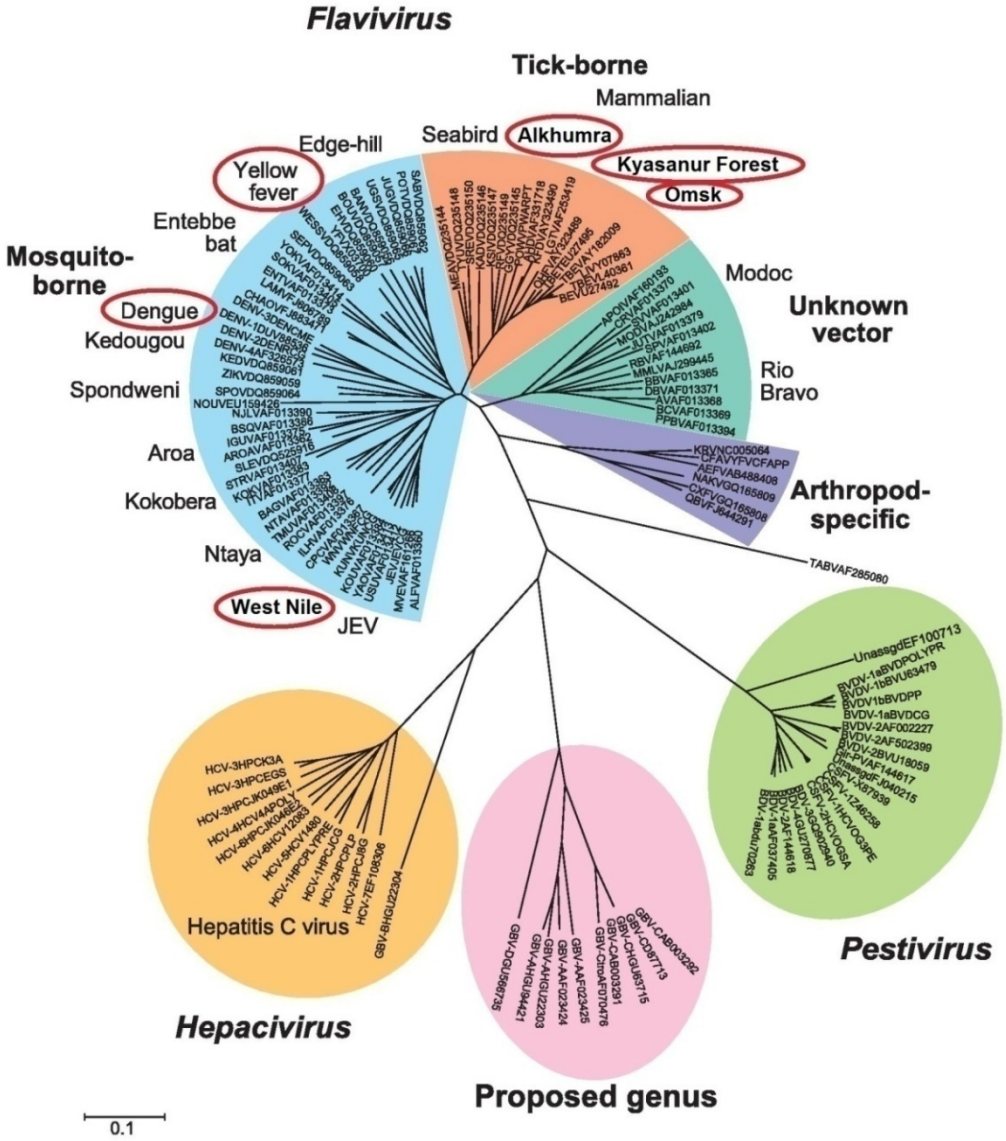

Figure 4: An unrooted phylogenetic tree based on the conserved sequences of RdRp (NS5 or NS5B) of members of the family *Flaviviridae* constructed by neighbor-joining method of amino acid p-distances using MEGA 4. Note that the Entebbe virus group comprising Entebbe bat virus, Sokoluk virus and Yokose virus have no known vector, they but group phylogentically within the mosquito-borne flaviviruses. Nounané, Chayoang, Ngoye and Lammi viruses are not currently assigned to a specific virus group but also group within the mosquito-borne viruses. The species highlighted in red ellipse represent members of the *Flaviviridae* which are known as causative agents of hemorrhagic fevers (customized according to Simmonds *et al.*, 2012).

Table 1: List of mosquitoes associated carrying VHFs (de Rodaniche and Galindo, 1957; Rodhain and Rosen, 2001; web sources 7, 10).

Mosquito species	Hosted virus	Mosquito species	Hosted virus
Aedes aegypti	YFV/WNV/DENV	*Anopheles hermsi*	WNV
Aedes africanus	YFV	*Anopheles punctipennis*	WNV
Aedes albopictus	YFV/WNV/DENV	*Anopheles quadrimaculatus*	WNV
Aedes atlanticus/tormentor	WNV	*Anopheles walkeri*	WNV
Aedes atropalpus	WNV	*Conquillettidia pertubans*	WNV
Aedes canadensis	WNV	*Culex apicalis*	WNV
Aedes cantator	WNV	*Culex bahamensis*	WNV
Aedes cinereus	WNV	*Culex coronator*	WNV
Aedes condolescens	WNV	*Culex erraticus*	WNV
Aedes dorsalis	WNV	*Culex nigripalpus*	WNV
Aedes dupreei	WNV	*Culex pipiens*	WNV
Aedes epactius	WNV	*Culex quinquefasciatus*	WNV
Aedes fitchii	WNV	*Culex restuans*	WNV
Aedes fulvus pallens	WNV	*Culex salinarius*	WNV
Aedes grossbecki	WNV	*Culex stigmatosoma*	WNV
Aedes infirmatus	WNV	*Culex tarsalis*	WNV
Aedes japonicus	WNV	*Culex territans*	WNV
Aedes leucocelaenus	YFV	*Culex thriambus*	WNV
Aedes melanimon	WNV	*Culiseta impatiens*	WNV
Aedes nigromaculis	WNV	*Culiseta incidens*	WNV
Aedes niveus	DENV	*Culiseta inornata*	WNV
Aedes polynesiensis	DENV	*Culiseta melanura*	WNV
Aedes provocans	WNV	*Culiseta morsitans*	WNV
Aedes scutellaris	DENV	*Culiseta perticeps*	WNV
Aedes sollicitans	WNV	*Deinocerites cancer*	WNV
Aedes squamiger	WNV	*Haemagogus equinus*	YFV
Aedes sticticus	WNV	*Haemagogus gorgasi*	YFV
Aedes stimulans	WNV	*Haemagogus lucifer*	YFV
Aedes taeniorhynchus	WNV	*Haemagogus spegazzinii*	YFV
Aedes triseriatus	WNV	*Mansonia titillans*	WNV
Aedes trivittatus	WNV	*Orthopodomyia signifera*	WNV
Aedes vexans	WNV	*Psorophora ciliata*	WNV
Anopheles atropos	WNV	*Psorophora columbiae*	WNV
Anopheles barberi	WNV	*Psorophora ferox*	WNV
Anopheles bradleyi/crucians	WNV	*Psorophora howardii*	WNV
Anopheles franciscanus	WNV	*Sabethes chloropterus*	YFV
Anopheles freeborni	WNV	*Uronataenia sapphirina*	WNV

1.6 Antiviral research and prevention against viral hemorrhagic fevers

1.6.1 Virus-targeting antivirals

Ribavirin

Ribavirin (RBV) is a broad-spectrum chemical compound with efficacy against many DNA and RNA viruses, *in vitro* and *in vivo* (Sidwell *et al.*, 1972; Graci and Cameron, 2006). The mechanisms of antiviral activity are based on its ability to inhibit inosine monophosphate dehydrogenase, a crucial enzyme responsible for the synthesis of GTP *de novo*. Other targets for its antiviral effect have been described, such as capping, translational efficiency of viral mRNA, and a suppressive effect on the viral polymerase activity (Chung *et al.*, 2013). Against hantaviruses, the mechanism of RBV seems to be more likely virus-unspecific. RBV was proved also as a potent mutagen of viral RNA (Crotty *et al.*, 2001, 2002; Jonsson *et al.*, 2005; Chung *et al.*, 2007, 2013).

RBV was reported to play a role in the immune response by down-regulation of interleukin-10 (IL-10)-producing Treg 1 cells, which could inhibit the conversion of $CD4^+$ $CD25^-$ $FOXP3^-$ naive T cells into $CD4^+$ $CD25^+$ $FOXP3^+$ adaptive Treg cells to maintain Th1 cell activity. However, the RBV-induced immune response against hantavirus infection is not yet fully discovered as well as the mutagenesis induced by RBV and its influence on next generations of virions (Kobayashi *et al.*, 2012).

The antiviral activity of RBV against HFRS and HCPS associated hantaviruses was tested *in vivo* and *in vitro*, as well (Huggins *et al.*, 1986; Chung *et al.*, 2013; Safronetz *et al.*, 2011). RBV-treated suckling mice infected by HTNV showed significantly higher survival rate than the placebo control group (Huggins *et al*., 1986). A double-blind placebo-controlled test of HFRS Chinese patients resulted in sevenfold lower morbidity and fatal ending in RBV-treated group (Huggins *et al*., 1991). The rates of oliguria and renal insufficiency are lower after the treatment by RBV, which increases the survival rate. In contrary, RBV used against HCPS seems to be more ineffective (Chapman *et al.*, 1999; Mertz *et al.*, 2004; Chung *et al.*, 2013). Interestingly, RBV appears to be sufficiently active in treatment of HCPS caused by ANDV (Safronetz *et al.*, 2011).

RBV is associated with potentially serious side effects, such as anemia and the teratogenicity if used in pregnant women. Some studies suggest that there is no significant difference in the frequency of adverse events (Mertz *et al*., 2004). Severe anemia appears in about 10% of treated patients, therefore a monitoring of hemoglobin is required. In cases of anemia, the reduction of RBV doses is needed, but this can cause compromising of sustained virologic response. Anemia is most probably a consequence of RBV accumulation in erythrocytes due to straight unidirectional transport through the membranes. Nowadays, the only prevention of RBV-induced anemia is the concomitant administration of erythropoietin (Russmann *et al*., 2006).

RBV has been successfully used to treat hamsters infected by YFV when it was administered up to 2 days after infection (shortly before infecting the liver). On the other hand, in non-human primates has shown no results (Monath, 2008). RBV was also tested against Dengue virus infection in its vector *Aedes aegypti* mosquitoes which were infected with Dengue-2 virus and treated by RBV at a dose of 0.3 mg/ml via artificial feeding technique. In mosquitoes treated with RBV, the infection was reduced.

Lactoferrin

Lactoferrin (LF), an iron-binding glycoprotein, besides of antibacterial and antifungal effect was reported to have a broad antiviral activity (Bullen and Armstrong, 1979; Masson *et al*., 1969; Yi *et al*., 1997). It has been demonstrated that LF also inhibits hantavirus infection *in vitro* and *in vivo* (Murphy *et al*., 2000; 2001).

The antiviral effects of LF against hantaviruses was compared with those of RBV in study which was performed on Vero E6 cells infected by Seoul virus (SEOV). Post infection administration of 100 μg/ml of RBV inhibited the number of foci by 97.5%. Four hundred μg/ml of LF reduced the number of foci by 85% in comparison with cells of the control group. In cells pretreated with LF, the number of foci initiated to increase from 24 h post infection (hpi). LF inhibited viral shedding at 24 hpi, but not after 48 hpi (Murphy *et al*., 2001). Therefore, LF obviously inhibits an early phase of infection, most probably adsorption as indicated in another supportive study (Murphy *et al*., 2000). Accordingly, the inhibition of hantavirus glycoprotein (G2) expression was observed. By 48 hpi, the expression of G2 was increased in both, the control and LF

pretreated cells. The complete G2 inhibition was detected only in cells treated with the combination of LF/RBV from 12 hr on. The inhibition of adsorption theory is supported also by the fact that LF does not inhibit the expression of hantavirus G2 and N protein when the infection is established only in cell cultures (Murphy *et al*., 2001).

On the other hand, RBV actively inhibits viral protein expression within the cell and does not inhibit viral adsorption (Huggins *et al*, 1984; Streeter *et al*., 1973). RBV apparently inhibits viral transcription and reduces a massive release of virions from infected cells. Nevertheless, RBV solely is not able to eliminate the virus completely as well as LF. Both, RBV and LF gave significantly higher survival rates in test *in vivo*. RBV administered 1 hpi to mice at dose of 50 and 25 mg/kg gave 81.8 and 68.8% survival rates, respectively (Murphy *et al*., 2001). These results are well experimentally supported by other studies, too (Huggins *et al*., 1991). LF administered with dose of 160 mg/kg to mice 1 day prior to hantavirus inoculation had a survival rate of 70%. Double administration of LF enhanced the survival rate. The 160 and 40 mg/kg double administration resulted in 94.1% and 85.7% survival rates, respectively. The difference between single and double administration of LF could be probably due to insufficient adsorption in the single dose or an accumulative effect of LF in the body from the two administrations. Another reason of this difference could be a certain period of time which is necessary for activation of immune system by LF. It has been demonstrated that LF enhances cytotoxic activities of monocytes and NK cells (Murphy *et al.*, 2001).

Favipiravir

Favipiravir (Avigan; T-705; 6-fluoro-3-hydroxy-2-pyrazinecarboxamide) is an antiviral drug selectively inhibiting the RNA-dependent RNA polymerase mainly of influenza virus. Its efficacy against the hantaviruses, Maporal virus, DOBV and Prospect Hill virus was also reported *in vivo* in mice and hamsters. Its activity *in vitro* against these hantaviruses was in the range of 5–30 μg/ml (32–191 μmol/l), as calculated by results of FFU reduction assays. (Gowen *et al*., 2007; 2010; Buys *et al*., 2011). Favipiravir decreased detection of viral RNA and reduced infectious titers of SNV and ANDV *in vitro*. For both, the EC50 was calculated at ≤5 μg/ml (≤31.8 μM). In hamsters infected with ANDV, favipiravir reached 100% of effectiveness at

preventing lethal HCPS when hamsters were administrated with favipiravir on or before day 4 post exposure. In contrast, animals of the placebo group demonstrated breathing difficulties on day 6 or 7 post infection leading to severe respiratory distress with a fatal outcome by day 9 (Buys *et al*., 2011; Safronetz *et al*., 2013).

ETAR

1-β-D-ribofuranosyl-3-ethynyl-[1,2,4]triazole (ETAR) is a novel, nucleoside analogue. ETAR as well as RBV is a 3-substituted 1,2,4,-triazole-β-riboside, but with altered steric and hydrogen bonding capacity. Its mechanism is based on inosine monophosphate dehydrogenase inhibition with reduction of GTP pools, which was combined with residual complementary activity possibly affecting the L protein (Kumarapperuma *et al*., 2007; Goundry *et al.*, 2003).

The antiviral activity of ETAR against HTNV and ANDV as representatives of HFRS and HCPS was approved. The EC50 values for HTNV and ANDV according to FFU-reduction assay were 10 µmol/l and 4.4 µmol/l, respectively. ETAR was not toxic to Vero E6 cells up to a concentration of 880 µmol/l. Moreover, ETAR protected suckling mice from HTNV infection at similar degree as RBV. The evaluation of ETAR in the suckling mice model infected by HTNV showed that the *in vivo* antiviral activity of ETAR at the 12.5 and 25 mg/kg doses was similar to that of 50 mg/kg RBV (Chung *et al*., 2008).

As shown previously, RBV is responsible for increased frequency of errors during replication of the HTNV leading to increased mutation frequency. Although ETAR is structurally similar to RBV, a comparison to the placebo-treated HTNV group showed no significant change in mutation frequency caused by ETAR. The metabolites of ETAR accumulate to lower concentrations in cells than the metabolites of RBV, which means that ETAR metabolites interact more potently with targets than the metabolites of RBV. ETAR is not expected to induce mutations probably due to its lack of pseudo-base pair presence (Chung *et al*., 2008).

1.6.2 Virus fusion inhibition

The target of interest of another study was G2 envelope glycoprotein, which plays a role of the viral fusion protein. It seems to be similar with other molecules of class II fusion proteins, as suggested *in silico* and *in vitro* analyses (Cifuentes-Muñoz *et al.*, 2011; Tischler *et al.*, 2005). Its ectodomain is composed of three domains which are connected by a stem to the anchor in the viral envelope. It has been shown, that these fusion proteins could be inhibited by protein fragments spanning domain III (DIII) and the stem region. For this reason, recombinant ANDV DIII and stem peptides were synthesized and expected to inhibit membrane fusion and cell entry. Combination of DIII and the C-terminal part of stem region inhibited the infection of Vero E6 by ANDV up to 60% during the endosomal route of ANDV. When fusion of ANDV occured at the plasma membrane, infection was inhibited over 95%. According to these results, a strategy of using hantavirus stem fragments may obviously inhibit fusion of similar viruses within the same genus (Barriga *et al.*, 2016).

1.6.3 Immunotherapy

Presently, there are no published reports of controlled clinical use of immunotherapy for HFRS and HCPS in humans. Some studies in animal models (hamsters, mice and rats) indicated that passive administration of neutralizing antibodies (Abs) or polyclonal sera to HTNV can sufficiently protect animals from disease involved with the same species of virus (Zhang *et al.*, 1989; Arikawa *et al.*, 1992; Xu *et al.*, 2002). Anti-HTNV G2-specific neutralizing Abs administrated 4 dpi sufficiently protected hamsters and up to 2 dpi protected suckling mice from lethal outcome (Liang *et al.*, 1996; Xu *et al.,* 2002). Post-exposure administration of neutralizing Abs was demonstrated against HCPS-causing hantaviruses, as well. Immune plasma obtained from HCPS patients infected by ANDV and SNV protected hamsters and deer mice infected by homologous virus, respectively (Custer *et al*., 2003; Medina *et al.*, 2007).

1.6.4 Host-targeting antivirals

Corticosteroid therapy

High levels of proinflammatory cytokines, especially TNF-α were detected in sera of patients with HFRS and HCPS. TNF-α is released by neutrophils, NK cells, $CD8^+$ T cells as well as DC and macrophages infected by a hantavirus (Schönrich *et al*., 2015; Kilpatrick *et al*., 2004). An immunomodulatory treatment was firstly performed and evaluated during the Korean war, when oral or intramuscular application of corticoids reduced lethal cases of HFRS due to the shock, but the mortality was not decreased at all (Sayer *et al.*, 1955).

A retrospective analysis of 22 HCPS patients in Chile noted that high-dose methylprednisolone treatment reduced mortality during the shock (Tapia *et al*., 2000). Another study involved 60 Chilean patients with HCPS caused by ANDV. This study reported a phase 2, double-blind, placebo-controlled clinical trial to evaluate the parameters such as the safety and the efficacy of intravenously applied methylprednisolone in patients with HCPS in Chile. The treatment of HCPS with high-dose methylprednisolone seems to be safe, but it is not recommended for clinical use, because there was no significant difference in lethal outcome between the methylprednisolone recipients (8 of 30 patients - 27%) and placebo recipients (12 of 30 patients - 40%) (Vial *et al*., 2013).

Host-cell hantavirus-binding receptor inhibitors

Pathogenic hantaviruses attach to the surface of host cells using their $\alpha_v\beta_3$ integrins. For this reason, a couple of synthesized cyclic nonapeptides, CLVRNLAWC and CQATTARNC were designed, and found to inhibit SNV infection *in vitro* at a 4:1 nanoparticle-to-virus ratio (9.0% to 32.5% and 27.6% to 37.6%, respectively). CQATTARNC used at a 20:1 ratio, inhibited infection by 50% (Hall *et al.*, 2008).

Another peptidomimetic compounds were chosen on the base of their molecular structure and possible ability to bind $\alpha_v\beta_3$ cell receptor. Forty nine peptidomimetic molecules in the first round and 68 molecules in the second round of screening with

antihantavirus effect in the two thousand lower micromolar range were identified. In result, a unique set of chemical compounds for the next phases of the drug discovery development was obtained. Their antiviral potential needs to be refined and supported by *in vivo* studies (Hall *et al.*, 2010).

Therapy via blocking of bradykinin B2 receptor

Another promising idea for the therapy of hantavirus diseases is the use of bradykinin receptor antagonists. Increased capillary permeability and vascular leakage are typical for all hantavirus infections. Complement activation seems to be linked to vascular changes in PUUV infections. The mechanisms behind the changes of vascular permeability after hantavirus infection are obviously a multifactorial event which is not yet completely analyzed. It has been found that hantaviruses are responsible for increased activation of the kinin-kallikrein system during the infection of endothelial cells, resulting in the liberation of bradykinin (Bossi *et al.*, 2004; Golias *et al*., 2007; Taylor *et al*., 2013).

Bradykinin is a nonapeptide binding bradykinin B2 receptor in role of an inflammatory mediator which is responsible for a dilatation of the blood vessels, increased vascular permeability and subsequently causes the blood pressure to fall. Icatibant is a peptidomimetic drug which is a selective antagonist of bradykinin B2 receptors. Icatibant blocks the binding of bradykinin to the bradykinin B2 receptor by binding to this receptor itself (Taylor *et al*., 2013).

A case report described a 37-year-old Finnish male patient with severe PUUV infection successfully treated with a single dose of icatibant (Antonen *et al.,* 2013; Vaheri *et al*., 2014). A report of another case, a 67-year-old female patient with severe HFRS caused by PUUV described a patient with a malignant chronic lymphoproliferative disease mostly affecting the spleen. In addition, patients' blood disease was morphologically considered as either atypical chronic lymphotic leukemia (CLL) or splenic marginal zone lymphoma. The 2 day delay between the doses the icatibant had no significant role in the recovery. Although this patient did not die, the icatibant did not play the role in recovery. Icatibant was not sufficient probably due to an extremely severe case of PUUV infection. It can be useful to note that one of the

predictions of severity of disease is a spleen with abnormalities. Nevertheless, the bradykinin B2 receptor antagonist icatibant is surely worth a further study as a target in the treatment of severe hantavirus infections (Laine *et al*., 2015).

1.6.5 Vaccines

There are no Food and Drug Administration (FDA)-licensed vaccines for HFRS or HCPS. Some vaccines based on use of immunoactive inactivated virus particles are in use in Far East, particularly in China and Korea. Other approaches of vaccine development were also studied and evaluated. Recently, some excellent review articles about vaccines against hantaviruses have been already published (Maes *et al.*, 2009; Schmaljohn, 2009; 2012; Krüger *et al.*, 2011), so we focused mostly on the most recent highlights. Flaviviruses bring interesting challenges in vaccine development. On the one hand, a vaccine against YFV (YF-Vax) works perfectly for a decade since it was developed. On the other hands, it is still unsuccessful to develop a sufficient immunoprophylaxis against DENV.

Live attenuated vaccines

Yellow fever 17D vaccine (YF-Vax) is a live-virus vaccine that has been used since 1938. A single dose is enough to provide a lifelong immunoprophylaxis of most people. The vaccine is recommended for people aged $\geq$ 9 months who are travelling to or living in areas at permanent risk for YFV transmission in Africa and South America. Side effects to YFV vaccine are mild and can include headaches, muscle aches, and subfebrilia. Serious events following YFV vaccination are rare. It could be life-threatening allergic reaction, disease of nervous system, and/or disease of internal organs. It should be a major care of healthcare provider or travel health clinic provider to look for serious adverse events (web source 11, 12).

Inactivated virus vaccines

In the last years some concerns have arisen about safety of the YFV 17D vaccine, mainly viscerotropic adverse events, which have a case-fatality rate of 64%. A non-replicating vaccines based on cell cultures would definitely not cause these adverse events, and could be used in persons of a risk group with some pracautions to use the live vaccine. There has been developed a whole virion vaccine from the 17D strain inactivated with beta-propiolactone, and adsorbed to aluminium hydroxide. It highly immunized mice, hamsters, and macaques. Hamsters and macaques were protected after a single dose of the vaccine by the similar titer of antibodies to those elicited by the 17D vaccine. Two doses of inactivated vaccine caused higher titer of antibodies than only a single dose of live 17D vaccine (geometric mean titer 20,480 vs. 1940, respectively) A clinical trial of the inactivated vaccine (XRX-001) has been started (Monath *et al.*, 2010).

Anti-hantavirus inactivated vaccines used in China and Korea are generally inactivated by formalin or β-propiolactone. These vaccines are aimed to protect against the hantaviruses HTNV, SEOV and PUUV, which are causing most of the cases of HFRS (Zhang *et al.*, 2010). Hantavax™, a formalin-inactivated vaccine developed in Korea consists of HTNV amplified in mouse brains. It is commonly used since 90's in Korea and China. Hantavax™ showed immunogenicity lasting at least two years with a three-dose schedule. The protective neutralizing antibody response showed to be sufficient just after third boosting dose (Song *et al.*, 2016).

Except of China and Korea, the research of anti-hantavirus vaccines was established also in Russia. An inactivated bivalent PUUV/DOBV vaccine consisting of the hantavirus strains PUUV Ufa-97 and DOBV-Aa Lipetzk-06 was developed. The aluminium hydroxide was used as adjuvant. This vaccine showed a significant neutralizing antibody activity against both PUUV and DOBV in immunized BALB/c mice. This bivalent vaccine against PUUV and DOBV passed pre-clinical tests under the Russian control authority institution and seems to be a promising approach in prevention against these species of hantaviruses (Krüger *et al*., 2011).

Recently, there are no studies about using of live attenuated hantaviruses for humans. Meanwhile, it has been proposed that a genetic reassortant of pathogenic and

non-pathogenic virus species could be a feasible vaccine development. A particle from the S and L segments of Prospect Hill virus and the M segment of PUUV was constructed. This virus particle interacted with elements of the innate immune system *in vitro* as Prospect Hill virus, but because of the PUUV origin of the M segment is expected to induce anti-PUUV neutralizing immune response (Handke *et al*., 2010).

Chimeric molecular vaccines

Non-replicating adenovirus vectors showed to be good carriers for a development of recombinant vaccines against hantaviruses ANDV and SEOV as representatives of HCPS and HFRS causing hantaviruses. Adenovirus expressing ANDV N, G1 or G2 proteins sufficiently protected the hamsters against lethal outcome of infection with ADNV (Safronetz *et al.*, 2009). Another model of a replication-competent recombinant canine adenovirus type 2 expressing the G1 protein of SEOV (rCAV-2-G1) in BALB/c mice was evaluated. Sera from immunized mice contained antibodies which specifically recognized SEOV and neutralized it *in vitro*. The recombinant virus completely protected the animals against a lethal challenge with the highly virulent strain of SEOV-CC-2 (Yuan *et al*., 2009).

Development of an effective vaccine against DENV has met a couple of challenges. The DENV-host interaction of infections is unique and associated with severe disease as a result of sequential DENV infection, viral immune evasion, host antibody enhancement, host immune activation, and genetic predisposition. It is hard to identify markers of protective immunogenicity, the target of potential role of antibodies when a vaccine fails, and how could have impacted a large-scale vaccination on the evolution of wild-type DENV (Thomas and Endy, 2011). However, there has been developed a promising live-attenuated tetravalent chimeric vaccine, Dengvaxia, recently approved by the WHO and licensed in four DENV-endemic countries. The National Center Institute of Allergy and Infectious Diseases (NIAID), USA and Takeda, developed another two DENV vaccines, which are entering phase III testing. Dengvaxia is composed of four yellow fever 17D-DENV chimeras, the NIAID vaccine contains three mutagenized DENV and one DENV2/4 chimera. Takeda vaccine is built of attenuated DENV 2 and three DENV 2-DENV chimeras. Dengvaxia should protect one

after three doses for one year, but it is not recommended to vaccine suspectible and too sensitive individuals. The NIAID vaccine is more promising possibility for travelers with only one dose required for a full protection against DENV-2 challenge. The protection as well as safety of the Takeda vaccine still remains to be demonstrated (Halstead and Aquiar, 2016).

Still in the part of chimeric vaccines, although there is no human WNV vaccine yet approved, it is worth to mention that several vaccine candidates are in development. However, despite the demonstration of strong protection in animal models for most of them, only a few candidates have tested in humans in early phase I clinical trials. They are Chimeric Vax-WNO2, Chimeric WN/DENV4-3´delta 30, Clinical trial VRC303, and WN-80E They could be officialy available after approximately 3 year from now, in the best case (Brandler and Tangy, 2013).

Virus-like particles

Virus-like particles (VLPs), such as hepatitis B virus and polyomavirus core particles, are viral proteins carrying foreign epitopes (Ulrich *et al.*, 1998). HTNV-VLPs by co-expressing HTNV N protein and G1 and G2 glycoproteins in Chinese hamster ovary (CHO) cells were generated. Then, intramuscular and subcutaneous administrations of HTNV-VLPs were compared for the ability to induce a specific immune response against HTNV infection in mice. The vaccination with HTNV-VLPs resulted in the induction of higher levels of specific cellular immune response to N protein in contrast with inactivated vaccine (Li *et al*., 2010). It has been shown that more species of hantaviruses (ANDV and PUUV) are potent to form VLPs just from G1 and G2 glycoproteins which are pleomorphic and expose protrusions. The viral nucleoprotein was not required for particle formation. These characteristics can be used for inducing of specific immune response for different species of hantaviruses (Acuña *et al*., 2014).

DNA vaccines

Different types of DNA vaccines against HTNV, SEOV, PUUV, ANDV and SNV using linear DNA, plasmid DNA and alphavirus replicons carrying genes for N protein and/or glycoproteins have been made. Interestingly, their immunogenicity apparently differs in different animal models. The M segment of ANDV was presented as immunogenic in non-human primates and rabbits but not in hamsters (Hammerbeck *et al*., 2009).

Three groups of nine volunteers were vaccinated with DNA vaccines for HTNV, PUUV or with a mixture of both vaccines expressing G1 and G2 genes of these viruses within the Phase I study. Hantavirus neutralizing antibodies were detected in five of nine and seven of nine persons who received all three vaccinations with the HTNV or PUUV DNA vaccine. In case of combined vaccine group, seven of the nine participants after all three vaccinations developed antibodies against PUUV. The three strongest responders to the PUUV vaccine had a strong neutralizing response to the HTNV, too. Both, HTNV and PUUV DNA vaccines were immunogenic, but when mixed, more individuals responded to the PUUV in contrast to the HTNV DNA vaccine (Hooper *et al*., 2014).

DNA vaccines protecting from hantaviruses causing HCPS were demonstrated on geese which were vaccinated with an ANDV DNA vaccine encoding the virus envelope glycoproteins for a purpose to produce neutralizing antibodies for use in humans because availability of convalescent plasma from survivors is very limited. Geese are supposed to produce IgY and alternatively spliced IgYΔFc, that can be purified at high concentrations from egg yolks. IgY lacks the mammalian Fc that can create antibodies in horses, sheep, and rabbits reactogenic in humans. All geese developed a high-titer neutralizing antibodies after second vaccination. It was shown by a pseudovirion neutralization assay (PsVNA) that high level of these neutralizing antibodies were maintained for over 1 year. Moreover, a booster vaccination resulted in higher levels of neutralizing antibodies (i.e., $PsVNA_{80}$ titers >100,000). The protective efficacy of the sera was proved in hamster model of lethal HCPS. It was shown that IgY/IgYΔFc purified from eggs transferred to hamsters subcutaneously starting 5 days after IM challenge with ANDV (25 LD_{50}) protected 7 of 8 hamsters. As it was shown,

DNA vaccine/goose platform is obviously a good candidate of preventing a lethal HCPS when administered post-exposure (Haese *et al.*, 2015). A paper reviewing antivirals and vaccines against hantaviruses is just published (Szabó, 2017).

1.7 Basic approaches to phylogenetic analyses

Phylogenetic trees in general, are a good tool to depict and explore evolutionary relationships between genes and organisms. They are comparable to a pedigree and show which genes or organisms are most closely related. They are called phylogenetic trees, because they resemble the tree structure and the terms refer to the various parts of this diagram (i.e. root, branch, node, and leaf). The leaves of a tree are the extant (existing) taxa, also called as operational taxonomic units (OTUs), because they represent a real information (nt or aa sequence data) with which we operate. Internal nodes are hypothetical taxonomic units (HTUs), because they emphasize that they are the hypothetical progenitors of OTUs. A group of leaves that share the same branch have a monophyletic origin and is called a cluster. In a case of unrooted tree, individual taxa are only placed to each other according to their evolutionary relationship without indicating the direction of the evolutionary process. There is no indication which node could be the ancestor of all OTUs. A tree can be considered as rooted if one or more of the OTUs form outgroup because they are most probably the most distantly related OTUs (i.e. outgroup rooting). The rest then forms the ingroup. The ingroup and outgroup are jointed by the root node (Vandamme, 2009).

To investigate the evolution and relationships between organisms, different kinds of data can be used. Taxonomy is still based largely on morphology. But the increasingly available molecular information, such as nucleotide or amino acid sequences are the reason why the use of molecular data for inferring phylogenetic trees has now gained interest among biologist of different disciplines, and this approach is often used in addition to morphological data to study evolutionary relationships in further detail (Vandamme, 2009).

The aim of molecular phylogenetic analyses is to infer the most reliable estimation of the real evolutionary relationships between genes of organisms. The evolution can be expressed as the accumulation of genetic changes within populations

over time (Hungnes *et al.*, 2000). For the purposes of molecular phylogenetic analyses, sequence information data is stored in databases such as the National Center for Biotechnology Information (NCBI), the National Library of Medicine (NLM), the European Molecular Biology Laboratory (EMBL) or the DNA Database of Japan (DDJ). Some organizations, such as NCBI Blast can provide a search for homologous sequences based on similarity scores. The first step after obtaining a dataset of sequences (nucleotide or amino acid) is to align them according to homology of each position of nucleotide or amino acid within the sequence dataset. Alignment is an essential prerequisite for further phylogenetic analyses (Vandamme, 2009).

The methods for constructing phylogenetic trees from molecular data can be grouped according to the kind of data they use, discrete character states or a distance matrix of pairwise dissimilarities, and according to the algorithmic approach of the method, either using a clustering algorithm resulting in only one tree estimate, or using an optimality criterion to evaluate different tree topologies (Vandamme, 2009).

Basically, character-state methods are able to use any set of discrete characters, which can be morphological characters, physiological properties, restriction maps, or sequence data. A comparison of sequences includes comparing each sequence position within the alignment. The sequence position is considered as “character”, and the nucleotides or amino acids at that position are the “states”. All character positions are analyzed independently, so each alignment column is considered as an independent realization of the evolutionary process (Vandamme, 2009).

Distance matrix methods start by calculating some measure of the dissimilarity of each pair of OTUs to create a pairwise distance matrix. Next, they infer the evolutionary relationships of the OTUs from the matrix. Distance methods discard the original character state of the taxa. As a result, the information required to reconstruct character state of ancestral nodes is lost. A great advantage of these methods is very fast analysis of large datasets. Disadvantage is a poor statistical support of results. Neighbor-joining (NJ) method is a good example. The clustering method used by this algorithm minimizes the length of all internal branches and thus the length of entire tree. At the beginning, NJ constructs a star-like tree which has no internal branches. In the first step, it put the first internal branch and then, one by one the others and calculates the length of the resulting tree (Vandamme, 2009).

"Maximum parsimony (MP)" and "Maximum likelihood (ML)" represent two character-state methods. The principle of MP is to infer an evolutionary history by use of the minimum number of mutations needed for a tree construction. This method is relatively simple and statistically consistent. The MP is used mainly in cases in which the evolutionary rate is relatively slow. A disadvantages of MP is the estimations of that the common character is inherited directly, so the method always underestimates the differences between distantly related taxons (Vandamme, 2009)..

On the other hands, ML methods have the advantage of using a statistical criterion because they consider the probability that a tree gave rise to the observed aligned sequences given a specific evolutionary model. The major disadvantage is an exhaustive search, which explores all possible trees, which could have been constructed, and thus the computing time, grows explosively as the number of data increases. ML requires a use of a mathematical model which estimates the probability of substitutions at each character in the alignment. It is mostly calculated by Bayesian Information Criterion (BIC) or Akaike Information Criterion (AIC) (Posada and Crandall, 2001; Hoff *et al.*, 2016). For this model is required a simple tree which can be quickly calculated for these purposes by Neighbor-Joining method. Probably one of the most important parameters of the phylogenetic trees is the choise of a proper mathematical model. It is probably even more important than a method for the tree construction itself. A mathematical model allows to correct the actual percentage differences between sequences, which are always bigger than directly calculated distances between sequences due to multiple hits (mutations during the evolution). When two sequences are very divergent, it is likely that, at a certain position, two or more mutations have occurred. We are able to see only actual differences at each position between the sequences, but with a mathematical model, we are able to estimate a number of mutations which there occurred in past until the final visible form (Posada, 2009).

Bayesian inference is in the cathegory of character-state methods, but different to Maximum Likelihood. It calculates with a prior distributions of data and posterior distributions of data. Prior distribution means that among all possible phylogenetic trees, each one has the same probability to be true. When data are put into a particular tree, it can result differently. For each of the trees, we will have a different probability. That is a posterior distribution. For the search of the best tree, there is commonly used

Markov Chain Monte Carlo (MCMC), a stochastic model using a hot chain and a cold chained to search the best tree. If we imagine a hot chain and a small chain as two robots searching the trees in a landscape made of hills and valleys, hot chain is searching among the highest hills and cold chain among those smaller ones. Each chain can accept the highest place of the hill and swap to another. When a hot chain finds the highest peak of the hill and another higher hill is close to him, it swaps there and continues in searching process. It is like the hot chain is melting the landscape differences of hills divided by valleys and is flattering the whole landscape. Probably for this is the hot chain called hot. The function of cold chain is opposite. We can set a number of chains more than just one hot and one cold. The point where the hot and cold chain meet together is the point where they found the best tree (Ronquist *et al*, 2009).

A widely used additive statistical supporting approach is the bootstrap analysis (or bootstrapping). Bootstrapping is a sampling method for estimating the statistical error in cases when the underlying sampling distribution is either unknown or difficult do derive analytically (Efron and Gong, 1983). The bootsrapping represents a useful tool to approximate the underlying distribution by resampling the original dataset. Particularly in phylogenetic analyses, non-parametric bootstrapping starts by replicating the datasets of the same size as the original by randomly resampling alignment columns with replacements from the original alignment and reconstruction phylogenetic trees for each of them. In each replication, the columns are chosen randomly, some of them can be chosen more times as well as some could not be chosen at all. The result represent the likelihood when the clades resulted the same in more replications in a row. As more bootstrap replications are set, so higher statistical support of the results is gained (Schmidt and Haesler, 2009).

2. AIMS OF THE STUDY

2.1 Aims of the study at the Department of Virus Ecology, Biomedical Research Center, Institute of Virology, Slovak Academy of Sciences, Bratislava, Slovakia

Research activities under the supervision of RNDr. Boris Klempa, DrSc:

Primary objectives:

- Molecular screening of small mammals' tissue specimens to a hantavirus infection.
- Identification of hantavirus species from hantavirus-positive specimens based on the L-segment hantavirus segment nucleotide sequences.
- Obtaining the S-segment and M-segment nucleotide sequences of the hantavirus genome from the hantavirus-positive specimens.
- Sequence and phylogenetic analyses of the newly obtained hantavirus strains.

Secondary objectives:

- Virus isolation from the tissues of newly identified hantavirus-positive specimens.
- Developing of a duplex RT-PCR assay using an internal positive control for the screening of hantaviruses.

2.2 Aims of the study at the Department of Biomedical Sciences, Section of Microbiology and Virology, Faculty of Biology and Pharmacy, University of Cagliari, Monserrato (CA), Italy

Research activities under the supervision of Prof. Alessandra Pani:

Primary objectives:

- Evaluation of the antiviral activity of selected aryl-piperazine derivates against viruses from the genera *Hantavirus* and *Flavivirus*, which are causative agents of hemorrhagic fever in humans.

Secondary objectives:

- Evaluation of the antiviral activity of selected aryl-piperazine derivates against viruses with a different type of genome (in comparison with the genera *Hantavirus* and *Flavivirus*) from the genera *Lentivirus* (family *Retroviridae*), *Enterovirus* (family *Picornaviridae*), *Orthopoxvirus* (family *Rhabdoviridae*), and *Vesiculovirus* (family *Rhabdoviridae*).
- Evaluation of the antiproliferative activity of selected aryl-piperazine derivates against different types of tumoral cells.

3. MATERIALS AND METHODS

3.1 Screening of small mammals, sequence and phylogenetic analyses of novel hantavirus strains

3.1.1 RNA extraction

We stored the animal tissue samples on the dry ice (then stored at -80°C) or on the ice with RNAlater Tissue Storage Reagent (then stored at -20°C). The total RNA of animals suspicious for hantavirus infection was extracted from lung tissues using the Quick Gene RNA tissue kit SII (RT-S2) (KURABO Industries Ltd., Osaka, Japan). Small pieces (15-30 mg, 1.5-2 mm^3) of animal tissues were homogenized in 500 µl of Lysis Buffer with 2-Mercaptoethanol using a QIAGEN TissueLyzer (30 Hz/5 min. × 2times). After centrifugation at 14,000 rpm/3 min./RT we transferred 385 µl of supernatant to a new 1.5 ml microtube with 175 µl of Solubilizaion Buffer. After 15 sec. vortex and brief spinning down we added 140 µl of 99% ethanol. After 1 min. vortex and brief spinning down we transferred the whole lysate to the cartridge in QG-Mini80 machine and made the lysate to flow through the membrane. We washed the membranes with 750 µl of Wash Buffer three times. Then, we extracted the total RNA in the last flow throught in 50 µl of Elution Buffer and stored at - 80°C.

3.1.2 Reverse transcription and RT-PCR reagents

10 µl (1-5 µg) of the total RNA was reverse transcribed into the cDNA with 1 µl (200 units) of M-MLV Reverse Transcriptase (Invitrogen™, Waltham, MA, USA) using 1 µl (250 ng) of random primers, 1 µl of 10 mM dNTP mix (pH7), 4 µl of 5 × First-Strand Buffer [250 mM Tris-HCl(pH 8.3 at room temperature), 375 mM KCl, 15 mM $MgCl_2$], 2 µl of 0.1 M DTT, and 0.1 µl of RNase OUT™ Recombinant Ribonuclease Inhibitor (40 units/µl) according to manufacturer's protocol. One fourth of the total volume of cDNA (5 µl) was used as a tamplate for the 1st round of PCR reactions. All the RT-PCRs within the study were performed by Maxima Hot Start PCR Master Mix (2X). PCR master mix (50 µl) contained Maxima Hot Start *Taq* DNA

polymerase supplied in 2X Hot Start PCR buffer, 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP and 4 mM Mg^{2+} (Thermo Scientific™, Waltham, MA, USA).

3.1.3 Hantavirus initial screening RT-PCR by targeting the virus L segment

The Pan-Hanta-PCR assay was developed to detect and identify the species of hantaviruses which are currently known and also those novel ones which are not discovered, yet. A set of degenerated primers (HANL-F1 and HANL-R1 for first round of PCR and HANL-F2 and HANL-R2 for nested PCR) were based on the alignment of available nucleotide sequences of different species of hantaviruses (HTNV, SEOV, DOBV, PUUV, TULV, SNV, and ANDV) and on their common highly conserved region of L segment (Klempa *et al.*, 2006).

The thermal cycling conditions of both, 1st Round of PCRs (40 cycles) and nested PCRs (cycles) were: 95°C for 4 min for initial denaturation / enzyme activation, 95°C for 30 sec, 53°C for 30 sec and 72°C for 30 sec, followed by one cycle of final extension for 5 min at 72°C.

3.1.4 RT-PCR for the sequencing of PUUV and DOBV partial S and M segments

After the initial screening and the species identification based on the partial L segment nucleotide sequences in BLAST, we used a different sets of primers for (semi-) nested RT-PCRs to obtain a maximum possible length of nucleotide sequences from S and M segments of both, PUUV and DOBV (Table 2). The primers were previously used in other studies, for S segment of PUUV (Bowen *et al.*, 1997), and for S segment (Sibold *et al.*, 2001) and M segment (Papa *et al.*, 1998) of DOBV. The program and the primers' annealing temperature of reactions were set according to mentioned studies and the manufacturers' protocol for Maxima Hot Start PCR Master Mix (2X) (Thermo Scientific™, Waltham, MA, USA).

Table 2: List of primers used within the study

Target	Primer name	Primer sequence 5′ → 3′	References
All hantaviruses			
L segment	HAN-L-F1	ATG TAY GTB AGT GCW GAT GC	(Klempa *et al.*, 2006)
	HAN-L-R1	AAC CAD TCW GTY CCR TCA TC	(Klempa *et al.*, 2006)
	HAN-L-F2	TGC WGA TGC HAC IAA RTG GTC	(Klempa *et al.*, 2006)
	HAN-L-R2	GCR TCR TCW GAR TGR TGD GCA A	(Klempa *et al.*, 2006)
PUUV			
S segment	Puu 1a	TAT GGI AAT GTC CTT GAT GT	(Bowen *et al.*, 1997)
	Puu 1b	GCA CAI GCA AAI ACC CA	(Bowen *et al.*, 1997)
	Puu 2a	CCI AGT GGI CAI ACA GC	(Bowen *et al.*, 1997)
	Puu 2b	AAI CCI ATI ACI CCC AT	(Bowen *et al.*, 1997)
	Puu 2a_I	CCI AGT GGY CAR ACA GC	(Bowen *et al.*, 1997)
M segment	PUU-M-2222F	CAT TTR GGY CAY TGG ATG GAT G	this study
	PUU-M-3123R	GTA GAR CCA TAA CAC ATY GC	this study
	PUU-M-2300F	TAT CCW TGG CAR ACA GCX GG	this study
	PUU-M-3111R	CAC ATY GCW GAR TCA CAT GC	this study
DOBV			
S segment	D113F	GAT GCA GAI AAI CAI TAT GAR AA	(Sibold *et al.*, 2001)
	D1162R	AGT TGI ATI CCC ATI GAI TGT	(Sibold *et al.*, 2001)
	D357F	TGC WGA TGC HAC IAA RTG GTC	(Sibold *et al.*, 2001)
	D955R	GCR TCR TCW GAR TGR TGD GCA A	(Sibold *et al.*, 2001)
M segment	DM1470F	CCI GGI TTI CAT GGI TGG GC	(Papa *et al.*, 1998)
	DM2029R	CCA TGI GCI TTI TCI KTC CA	(Papa *et al.*, 1998)
	DM1674F	TGT GAI RTI TGI AAI TAI GAG TGT GA	(Papa *et al.*, 1998)
	DM1990R	TCI GMT GCI STI GCI GCC CA	(Papa *et al.*, 1998)

For the M segment of PUUV, we used a new set of primers which were designed in Geneious software (Auckland, New Zealand). The annealing temperature of these primers was set at 55 °C for both, 1st Round and nested PCR, as well.

3.1.5 Methods *in silico*

Obtained PCR products were sequenced by Sanger method as a commercial service in Ecoli Ltd. (Bratislava, Slovakia). Screening of the rodent tissue samples from Germany was performed and resulting sequences were obtained by our collaborating partners (Bundeswehr Institute of Microbiology, Dept. Virology & Rickettsiology, Munich, Germany). After obtaining the first sequence data from the primary L segment

screening, we used SeqMan Pro (Lasergene software package, DNASTAR, WI, USA), to assemble two sequences (from both directions) and get one consensus sequence. SeqMan Pro is a useful tool which allows to visualize a sequencing signal with all errors (if they appeared) which had been not corrected automatically. In most of cases, these errors are easy to correct manually. An important step in consezus sequences making was to cut the terminal sequence information which corresponds to the primer sequence. SeqMan Pro was used for assembling S and M segment sequences, too. We used BLAST (Basic Local Alignment Search Tool), an online available tool which finds regions of similarity among a wide range of different biological sequences. The program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance.

Multiple alignments of nucleotide sequences were generated by the program MUSCLE (available within MEGA7). It is interesting to note, that the speed and accuracy of MUSCLE were compared with other programs aligning nucleotide sequences, such as T-Coffee, MAFFT, and CLUSTALW and MUSCLE achieved the highest or joint highest rank in accuracy in all tests (Edgar, 2004).

The estimation of the best substitution model is considered as a crucial step for good results particularly when Maximum Likelihood method is used for the construction of phylogenetic trees. For the estimation of the best substitution model we chose the Bayesian Information Criterion (BIC) method (Schwarz, 1978), which seemed to be appropriate for our purposes according to previously published studies (Nei and Kumar, 2000). The Best-fit model tests were performed in MEGA7.

All five phylogenetic trees in results below were performed in program MEGA7, as well. As a method for the inference of phylogeny Maximum Likelihood (ML) was chosen for all five cases using the best-fit substitution model according to the results of Model test for each tree separately. As a statistical support, we used a Bootstrapping with 1000 replications. Only the values of the results of bootstrapping ≥ 70 are shown on the branches, as we considered them as statistically significant. Additional sequence analyses- Sequence Identity Matrix, were performed in BioEdit software (Hall, 1999).

3.1.6 Virus isolation attempts

We tried to isolate infectious PUUV virus from RT-PCR-positive lung tissue samples from wild bank voles trapped in East Slovakia (Cat. No.: PUUV/SVK129/Mg, PUUV/SVK132/Mg, PUUV/SVK780/Mg). 60 µg of the tissue were suspended in Dulbecco's medium (DMEM) supplemented with 0.2% bovine serum albumin (BSA). Ratio: 60 µg tissue/ 600 µl DMEM/ 0.6 mg BSA. The tissues were homogenized with QIAGEN TissueLyzer (30 Hz/5 min. × 2times). Homogenized tissues were briefly centrifugated at low speed: 14,000 rpm/3 min./RT. Then, 400 µl of the supernatant was used for the inoculation of confluent VERO E6 cell cultures in 25 cm^2 flasks (three flasks for each sample). Cells with infectious inoculum were incubated at 37°C. We changed the medium after 90 min. and then in every one week interval (Figure 5).

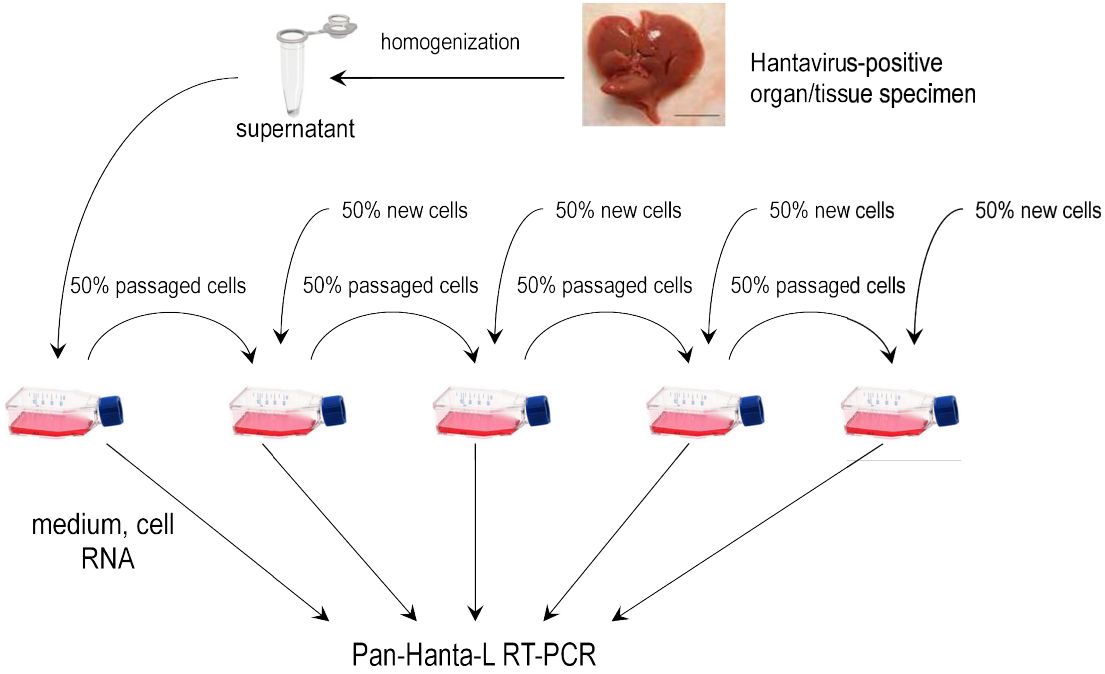

Figure 5: Scheme of the virus isolation attempts according to published procedure (Nemirov *et al.*, 1999).

We passaged the cells in every 2 week intervals into the new flasks by adding uninfected cells in half of the total amount (ratio 50:50) according to the published procedure (Nemirov *et al.*, 1999), which have been published particularly for the isolation of hantaviruses. Altogether, we made 4 passages in a period of 8 weeks. After

each cell passage, we stored the old medium digested by cells and the cells from the previous passage into the cell culture freezing medium at -80°C.

3.2 Bacteriophage MS2 as an internal control in duplex RT-PCR assay for the screening of hantaviruses

Bacteriophage MS2 (DSM13767) was purchased in DSMZ institute (Braunschweig, Germany). MS2 was cultivated in *E. coli* XL-10 Gold (Stratagene, La Jolla, CA) harboring F-plasmid by the double-agar-layer plaque cultivation technique (Ausubel *et al.*, 1999). We harvested the top agar layer with confluently destructed cells and put them into 4 ml of suspension medium (SM) buffer (0.1 M NaCl, 8 mM $MgSO_4$, 0.05 M Tris-HCl, pH 7.5, 0.01% [wt/vol] solid gelatin). The bacteriophage particles were isolated by low-speed centrifugation (4,000 × g/30 min./4°C) with 4 ml of chloroform. Then, we obtained the phage particles from the supernatant (cca 3 ml) and stored at -20°C. The PFU was calculated from the dilutions (10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}) of the MS2 phage stock using the double-agar-layer technique as previously. The phage MS2 stock solution contained 3×10^{11} PFU/ml. For the next purposes, the phage MS2 stock was diluted in SM buffer to the final concentration 6 x 10^4 PFU/ml.

In the first step of the hantavirus detection from wild animals we extracted a total RNA by a Quick Gene RNA tissue kit SII (RT-S2) (KURABO Industries Ltd., Osaka, Japan). Small pieces (15-30 mg or 1.5-2 mm^3) of random non-infectious animal tissue were added into 500 µl of Lysis Buffer with 2-Mercaptoethanol. To simulate the infectiousness we added 50 µl of Prospect Hill virus (PHV, strain: PH-1) suspension. For measuring the sensibility of the procedure we used different titers of PHV suspension (1 x 10^3, 1 x 10^2, 1 x 10^1 and 1 FFU/ml). Then we added 50 µl of the internal genomic control- MS2 phage solution (6 x 10^4 PFU/ml). The tissue with bacteriophages was homogenized by QIAGEN TissueLyzer (30 Hz/5 min. × 2times). After the procedure according to the manufacturer's protocol, we extracted the total RNA in 50 µl of Elution Buffer and stored at - 80°C. 10 µl (1-5 µg) of the total RNA was reverse transcribed into the cDNA with 1 µl (200 units) of M-MLV Reverse Transcriptase (Invitrogen™, Waltham, MA, USA) using 1 µl (250 ng) of random primers, 1 µl of 10 mM dNTP mix (pH7), 4 µl of 5 × First-Strand Buffer [250 mM Tris-

HCl(pH 8.3 at room temperature), 375 mM KCl, 15 mM $MgCl_2$], 2 µl of 0.1 M DTT and 0.1 µl of RNase OUT™ Recombinant Ribonuclease Inhibitor (40 units/µl). One fourth of the total volume of cDNA (5 µl) was used as a tamplate for the 1st round of PCR reactions.

We modified the original Pan-Hanta-PCR assay RT-PCR assay by adding a set of primers for the bacteriophage MS2 RNA (Table 3). We performed the RT-PCR reactions by Maxima Hot Start PCR Master Mix (2X).

Table 3: RT-PCR program:

1. Round PCR	Vol. [µl]
Maxima Hot Start PCR Master Mix (2X)	25
HANL-F1	5
HANL-R1	5
MS2: 2717F	5
MS2: 3031R	5
= 45 µl + 5 µl cDNA	

PCR program:		
95°C	4′	
95°C	30′′	
53°C	30′′	40 ×
72°C	30′′	
72°C	5′	
4°C	hold	

(Semi-) Nested PCR	Vol. [µl]
Maxima Hot Start PCR Master Mix (2X)	25
HANL-F2	5
HANL-R2	5
a) b) c)	5/5
H_2O	4
= 49 µl + 1 µl PCR product	

PCR program:		
95°C	4′	
95°C	30′′	
53°C	30′′	25 ×
72°C	30′′	
72°C	5′	
4°C	hold	

(Semi-) Nested RT-PCR was performed in three different combinations of primers for the Bacteriophage MS2 cDNA: a) MS2: 2781F / MS2: 3031R b) MS2: 2717F / MS2: 2989R c) MS2: 2781F / MS2: 2989R.

3.3 Screening of biological activity of selected chemical compounds

3.3.1 Preparation of stocks of the compounds

A novel series of seventeen aryl-piperazine derivates (2Cl3C1, 2Cl3C2, 2Cl3C3, 2Cl3C5, 2Cl3C6, 2Cl3C8, 2Cl3C9, 2Cl3C10, 2Cl3C13, 2Cl4C1, 2Cl4C4, 2Cl4C5, 2Cl4C6, 2Cl4C7, 2Cl4C8, 2Cl4C10, 2Cl4C12) synthesized by our Partners of the Medical University of Warsaw (Poland). Compounds were dissolved in DMSO (dimethyl sulfoxide). The volume of DMSO was calculated according to mass of the powder and a molar mass of each of the compounds, using the formula showed below (Figure 6), to obtain the final concentration of 100 mM. The molar mass was given together with compounds' molecular formulas (Appendix 1).

$$[\mu L]DMSO = \frac{\text{Mass [mg]}}{\text{Molar mass}} \times 10{,}000$$

Figure 6: A formula for calculation of the volume of DMSO for making of 100 mM stock solutions from solid chemical substances.

Compounds' solutions were mixed by vortex and briefly held in ultrasonicator to obtain a homogenous colloid solution. Full systematic names of the aryl-piperazine derivates are listed under the scheme of synthesis (Figure 7). The skeletal structure formulas of each compound are listed in Appendix 1.

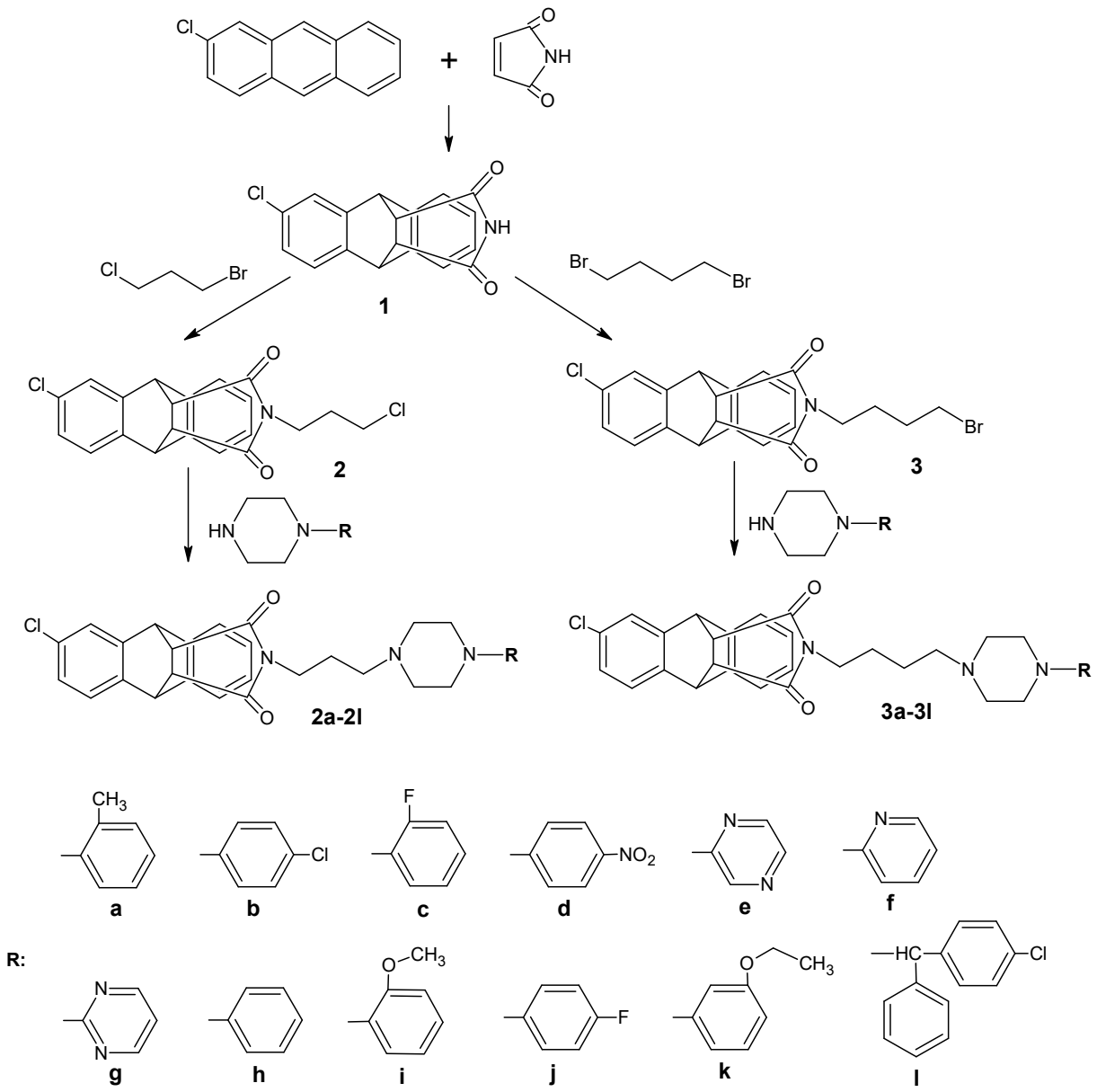

Figure 7: Scheme of synthesis of aryl-piperazine derivates used within the study.

1. 11-chloro-17-azapentacyclo[6.6.5.$0^{2,7}$.$0^{9,14}$.$0^{15,19}$]nonadeca-2,4,6,9(14),10,12-hexaene-16,18-dione

2. 11-chloro-17-(3-chloropropyl)-17-azapentacyclo[6.6.5.$0^{2,7}$.$0^{9,14}$.$0^{15,19}$]nonadeca-2,4,6,9(14),10,12-hexaene-16,18-dione

3. 17-(4-bromobutyl)-11-chloro-17-azapentacyclo[6.6.5.$0^{2,7}$.$0^{9,14}$.$0^{15,19}$]nonadeca-2,4,6,9(14),10,12-hexaene-16,18-dione

2a. 2Cl3C1; 11-chloro-17-{3-[4-(2-methylphenyl)piperazin-1-yl]propyl}-17-azapentacyclo[6.6.5.0^{2,7}.0^{9,14}.0^{15,19}]nonadeca-2,4,6,9(14),10,12-hexaene-16,18-dione

2b. 2Cl3C2; 11-chloro-17-{3-[4-(4-chlorophenyl)piperazin-1-yl]propyl}-17-azapentacyclo[6.6.5.0^{2,7}.0^{9,14}.0^{15,19}]nonadeca-2,4,6,9(14),10,12-hexaene-16,18-dione

2c. 2Cl3C3; 11-chloro-17-{3-[4-(2-fluorophenyl)piperazin-1-yl]propyl}-17-azapentacyclo[6.6.5.0^{2,7}.0^{9,14}.0^{15,19}]nonadeca-2,4,6,9(14),10,12-hexaene-16,18-dione

2e. 2Cl3C5; 11-chloro-17-{3-[4-(pyrazin-2-yl)piperazin-1-yl]propyl}-17-azapentacyclo[6.6.5.0^{2,7}.0^{9,14}.0^{15,19}]nonadeca-2,4,6,9(14),10,12-hexaene-16,18-dione

2f. 2Cl3C6; 11-chloro-17-{3-[4-(pyridin-2-yl)piperazin-1-yl]propyl}-17-azapentacyclo[6.6.5.0^{2,7}.0^{9,14}.0^{15,19}]nonadeca-2,4,6,9(14),10,12-hexaene-16,18-dione

2h. 2Cl3C8; 11-chloro-17-[3-(4-(phenylpiperazin-1-yl]propyl}-17-azapentacyclo[6.6.5.0^{2,7}.0^{9,14}.0^{15,19}]nonadeca-2,4,6,9(14),10,12-hexaene-16,18-dione

2i. 2Cl3C9; 11-chloro-17-{3-[4-(2-methoxyphenyl)piperazin-1-yl]propyl}-17-azapentacyclo[6.6.5.0^{2,7}.0^{9,14}.0^{15,19}]nonadeca-2,4,6,9(14),10,12-hexaene-16,18-dione

2j. 2Cl3C10; 11-chloro-17-{3-[4-(4-fluorophenyl)piperazin-1-yl]propyl}-17-azapentacyclo[6.6.5.0^{2,7}.0^{9,14}.0^{15,19}]nonadeca-2,4,6,9(14),10,12-hexaene-16,18-dione

2l. 2Cl3C13; 11-chloro-17-(3-{4-[(4-chlorophenyl)(phenyl)methyl]piperazin-1-yl}propyl)-17-azapentacyclo[6.6.5.0^{2,7}.0^{9,14}.0^{15,19}]nonadeca-2,4,6,9(14),10,12-hexaene-16,18-dione

3a. 2Cl4C1; 11-chloro-17-{4-[4-(2-methylphenyl)piperazin-1-yl]butyl}-17-azapentacyclo[6.6.5.0^{2,7}.0^{9,14}.0^{15,19}]nonadeca-2,4,6,9(14),10,12-hexaene-16,18-dione

3d. 2Cl4C4; 11-chloro-17-{4-[4-(4-nitrophenyl)piperazin-1-yl]butyl}-17-azapentacyclo[6.6.5.0^{2,7}.0^{9,14}.0^{15,19}]nonadeca-2,4,6,9(14),10,12-hexaene-16,18-dione

3e. 2Cl4C5; 11-chloro-17-{4-[4-(pyrazin-2-yl)piperazin-1-yl]butyl}-17-azapentacyclo[6.6.5.0^{2,7}.0^{9,14}.0^{15,19}]nonadeca-2,4,6,9(14),10,12-hexaene-16,18-dione

3f. 2Cl4C6; 11-chloro-17-{4-[4-(pyridin-2-yl)piperazin-1-yl]butyl}-17-azapentacyclo[6.6.5.0^{2,7}.0^{9,14}.0^{15,19}]nonadeca-2,4,6,9(14),10,12-hexaene-16,18-dione

3g. 2Cl4C7; 11-chloro-17-{4-[4-(pyrimidin-2-yl)piperazin-1-yl]butyl}-17-azapentacyclo[6.6.5.$0^{2,7}$.$0^{9,14}$.$0^{15,19}$]nonadeca-2,4,6,9(14),10,12-hexaene-16,18-dione

3h. 2Cl4C8; 11-chloro-17-[4-(4-(phenylpiperazin-1-yl]butyl}-17-azapentacyclo[6.6.5.$0^{2,7}$.$0^{9,14}$.$0^{15,19}$]nonadeca-2,4,6,9(14),10,12-hexaene-16,18-dione

3j. 2Cl4C10; 11-chloro-17-{4-[4-(4-fluorophenyl)piperazin-1-yl]butyl}-17-azapentacyclo[6.6.5.$0^{2,7}$.$0^{9,14}$.$0^{15,19}$]nonadeca-2,4,6,9(14),10,12-hexaene-16,18-dione

3k. 2Cl4C12; 11-chloro-17-{4-[4-(2-ethoxyphenyl)piperazin-1-yl]butyl}-17-azapentacyclo[6.6.5.$0^{2,7}$.$0^{9,14}$.$0^{15,19}$]nonadeca-2,4,6,9(14),10,12-hexaene-16,18-dione

Note: Only those variants of aryl-piperazine derivates which we have had available are listed. Full final skeletal structures of aryl-piperazine derivates are listed in Appendix 1 according to abbreviated symbols in bold (2ClXCX). Structure, activity, and relationship study is described in Discussion according to aryl-piperazine chemical groups (R = 2a-2l ∨ 3a-3l).

3.3.2 Propagation of cell cultures from frozen stocks and their maintenance

For virus cultivation and cytotoxicity evaluation we used the following ATCC animal cells: VERO 76, VERO E6, BHK-21, MT-4. For antiproliferative evaluation we used the ATCC human derived tumoral cell lines: HeLa, SK-MEL-28, SK-MES-1, DU 145, CCRF-CEM, CCRF-SB, WIL-2NS, and a non-tumoral control: CRL-7065 (Table 4). After recovery from the frozen stock in liquid nitrogen, before experimental testing, cells were split 2-3 times in order to allow them to reach their regular growth rate.

For the maintenance of VERO cells (VERO76/VERO E6) was used Eagles' minimum essential medium (EMEM, Lonza Group Ltd., Basel Switzerland) containing 25 mM HEPES (Thermo Fisher Scientific, MA, USA), 10% FBS, 1% L-glutamine, 1 mM sodium pyruvate (NaPy), 1% NEAA (non essential amino acids), and 0.1% Gentamicin. All cell lines were regularly passaged twice a week in a ratio of dilution calculated according to their optimal level of growth.

The baby hamsters' kidney (BHK-21) cells were passaged with Minimum essential medium with Earle's salts (MEM-E), L-glutamine, 1 mM NaPy, 25mg/L kanamycin, supplemented with 10% FBS.

For non-adherent tumoral cell lines, which grow in suspension (CCRF-CEM, CCRF-SB, WIL-2NS), RPMI 1640 medium supplemented with 4.5g/L glucose, 2 mM L-glutamine and 10% fetal bovine serum, was used. All the cell cultures were incubated at 37 °C in a humidified, 5% CO_2 atmosphere, and periodically checked for mycoplasma contamination.

Table 4: List of tumoral cell lines and non-tumoral control (CRL-7065), their origin and properties.

Cell name	Organism	Tissue	Morphology	Culture properties	Disease	Age	Gender
HeLa	*H. sapiens*	cervix	epithelial	adherent	Adenocarcinoma	31	female
SK-MEL-28	*H. sapiens*	skin	polygonal	adherent	Malignant melanoma	51	male
SK-MES-1	*H. sapiens*	lung	epithelial	adherent	Squamous cell carcinoma	65	male
DU 145	*H. sapiens*	prostate	epithelial	adherent	Carcinoma	69	male
CCRF-CEM	*H. sapiens*	peripheral blood	lymphoblast	suspension	Acute lymphocytic leukemia	4	female
CCRF-SB	*H. sapiens*	peripheral blood	lymphoblast	suspension	Lymphoblastic leukemia	11,5	male
WIL-2NS	*H. sapiens*	spleen	lymphoblast	suspension	Hereditary spherocytosis	5	male
CRL-7065	*H. sapiens*	skin, foreskin	fibroblast	adherent	Normal	newborn	male

3.3.3 Methods of screening and evaluation of the compounds' biological activity

Potential inhibitory activity of compounds against hantaviruses (PUUV and SEOV) was evaluated by Focus-reduction assay performed in 6-well plates (Figure 8). The concentrations of the evaluated chemicals were set from 30 μM, three times diluted to 10 μM, 3 μM, and 1 μM. Only for title compounds, the dilutions were 100 μM, 50 μM, 25 μM, and 12.5 μM for Ribavirin and 10 μM, 5 μM, 2.5 μM and 1.25 μM for 6-azauridine. When the old medium was removed, the VERO E6 cells (8×10^5cells/well) were infected by PUUV or SEOV (MOI = 0.0000375). The infectious virus suspension was diluted in the HBSS medium and virus adsorption to cells was allowed for one hour in CO_2 thermostat and agitated every 10 minutes.

Then, virus inoculums was removed and 2.5 ml of the overlay medium was poured into each well. The 60 ml of the overlay medium contained 30 ml of the 1% agarose (diluted in ultrapure water), 25.7 ml 2×EMEM, 750 μl HEPES, 3 ml FBS, and 600 μl PSN (penicillin, streptomycin, neomycin). When 6-well plate was used, only one chemical compound was possible to evaluate within the one multiwell plate. The rate of the replication was measured by Focus reduction assay.

The primary antibody- anti Malacky (TULV-Malacky genotype) obtained from a rabbit attached the N-protein of hantaviruses. The secondary antibody- goat anti-rabbit IgG (H+L) HRP Conjugate (Cat. No. 170-6515, Bio-Rad, Inc., CA, USA) was attached to the primary antibody. Then, the SuperSignal West Pico Chemiluminescent Substrate (Cat. No. 34080, Thermo Scientific, MA, USA) was added and the foci were visualized by use of ChemiDoc MP Imaging System (Bio-Rad, Inc., CA, USA).

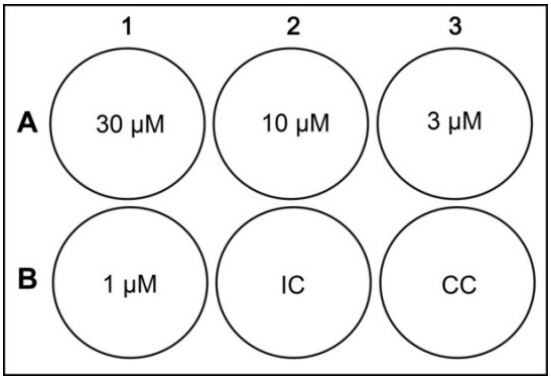

Figure 8: Universal scheme for the evaluation of chemical compounds against hantaviruses on 6-multiwell plate; IC- infection control; CC- cellular control.

Compounds' activity against Vaccinia virus (VV), Vesicular stomatitis virus (VSV), and Coxsackie virus, strain B5 (CVB-5) were determined by plaque reduction assay in infected monolayers of VERO 76 cells (Figure 9). VERO 76 cells were seeded in 24-well plates at a density of 2×10^5 cells/well and were allowed to form confluent monolayers by incubating overnight in growth medium at 37°C in a humidified CO_2 (5%) atmosphere. Then, monolayers were infected with appropriate virus dilutions (250 µl) to final 50-100 PFU/well. The viruses on VERO 76 were incubated at room temperature (normal atmosphere) for 90 minutes.

The unadsorbed virus was removed and replaced by Dulbecco's modified Eagle's medium (500 µl), supplemented with 1% inactivated FCS and 0.75% methyl cellulose, with or without added a serial of testing chemical compounds. A primary screening was performed with four dilutions of selected compounds (100 µM, 50 µM, 25 µM, and 12.5 µM). When some chemicals resulted as active, we repeated the screening by adding four dilutions more (6.25 µM, 3.1 µM, 1.6 µM, and 0.8 µM), as with a title compound at the beginning (Figure 9). Cultures were incubated at 37°C for 2 (VSV) or 3 (CVB-5, VV) days and then fixed with PBS containing 50% ethylalcohol and 0.8% crystal violet. The multiwell plates were at the end washed and dried on air. Concentrations resulting in 50% of inhibition (EC_{50}) were calculated according to number of plaques caused by a virus replication and were determined by a linear regression analysis.

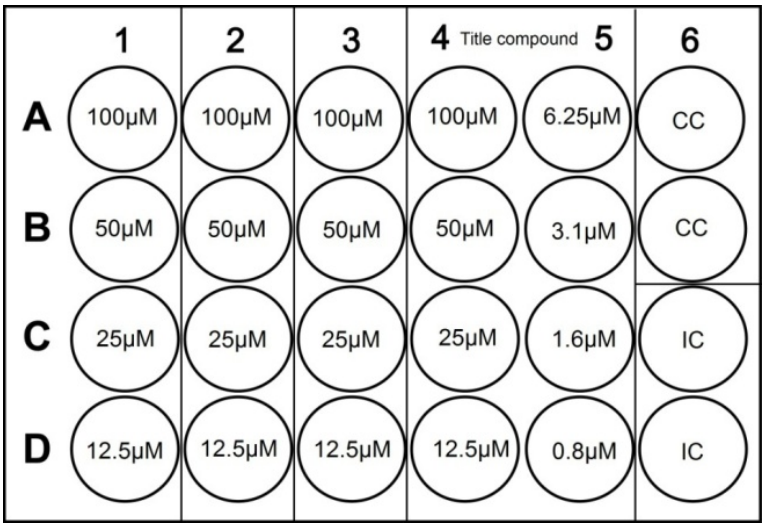

Figure 9: Universal scheme for the evaluation of chemical compounds against VV, VSV, and CVB-5 on 24-multiwell plate; IC- infection control; CC- cellular control; Numbers 1, 2, and 3 represent different random compounds.

The inhibitory activity test of compounds against Yellow fever virus (YFV) and Human Immunodeficiency virus type-1 (HIV-1) was based on virus-induced cytopathic effect by YFV in BHK-21 cells, and by HIV-1 in MT-4 cells. The infection was performed at the MOI= 0.01 for both, YFV and HIV-1. RPMI (50 µl) containing 1×10^4 MT-4 were added to a well (of 96-well plate) where was previously added RPMI (50 µl), with or without serial dilutions of tested compounds (Figure 10). The HIV-1 suspension (20 µl) containing 100 $CCID_{50}$ was then added. After 4 days of incubation, cell viability was determined by the MTT method. BHK-21 cells were seeded in 96-well plates at a density of 5×10^4 cells/well and were allowed to form confluent monolayers by incubating overnight in growth medium at 37°C in a humidified CO2 (5%) atmosphere. Cells were then infected with 50 µl of a proper virus dilution in serum free medium (MOI= 0.01). After one hour, MEM Earle's medium (50 µl), supplemented with inactivated fetal calf serum (FCS), 1% final concentration, with or without serial dilutions of tested compounds. After 3-4 days of incubation at 37°C, cell vialibility was determined by the MTT method.

To test the antiproliferative activity of compounds against tumoral cell lines grown in monolayer, 50 µl/ well of 2×10^5 cells/ml suspension of HeLa, SK-MEL-28, SK-MES-1, and DU 145 cells were seeded in 96-well plate. The non-tumoral cell control (CRL-7065) was seeded at a density of 4×10^5 cells/ml, 50 µl/well. Then, plates were incubated in complete growth medium (EMEM) for 24 hours. The medium was then removed, and the cells were incubated with 50 µl serum-free medium (RPMI 1640), with or without proper dilutions of chemical compounds. The evaluation of aryl-piperazine derivates inhibitory effect against tumoral cell lines grown as suspension (CCRF-CEM, CCRF-SB, WIL-2NS), RPMI medium with or without proper concentrations of chemical compounds was seeded (50 µl/well) in 96- well plate. Then, the cells were added at a density 10^4 cell/well. After 3-4 days of incubation at 37°C, cell vialibility was determined by the MTT method.

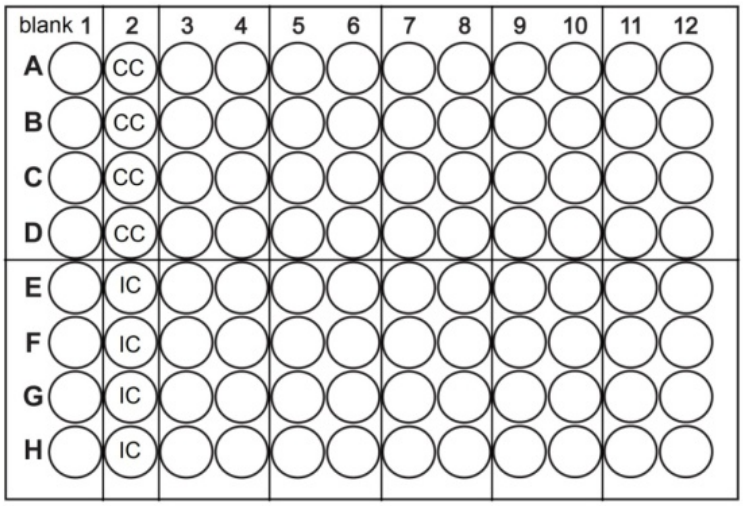

Figure 10: Universal scheme of distribution of 96-multiwell for MTT tests (used for: YFV/BHK-21; HIV-1/MT-4; HeLa, SK-MEL-28; SK-MES-1; DU 145; CCRF-CEM, CCRF-SB, WIL-2NS; CRL-7065). CC-cellular control; IC-infection control; Chemical substances were dilluted in medium to final concentrations: 100 μM, 20 μM, 4 μM, and 0.8 μM according to letter A, B, C, D, then E, F, G, H. Dilutions were distributed in pairs by numbers 3-4, 5-6. 7-8, 9-10, and 11-12.

4. RESULTS

4.1 Sequence and phylogenetic analyses of newly discovered strains of Puumala and Dobrava-Belgrade hantaviruses in Central Europe

4.1.1 Screening of small mammals from Central Europe region

A total of 578 small mammals representing three species were collected between the years 2007 - 2012 in Slovakia, Czech Republic and Germany. Among them, 523 were the *Clethrionomys* (*Myodes*) *glareolus* species (family *Muridae*, subfamily *Arvicolinae*) (408 from Germany: Bavarian Forest- Iggensbach, Elsenthal, Rachel, and Lackenber, Spessart Forest-Wiesenfeld; 72 from Slovakia: Šaštín, Morvaský sv. Ján, and Košice-Čermeľ; and 43 from Czech Republic: Jelení vrch, Prachatice, Žíchovec, Krušné hory, Beskydy, Blučina, and Tešetice). (Figure 11).

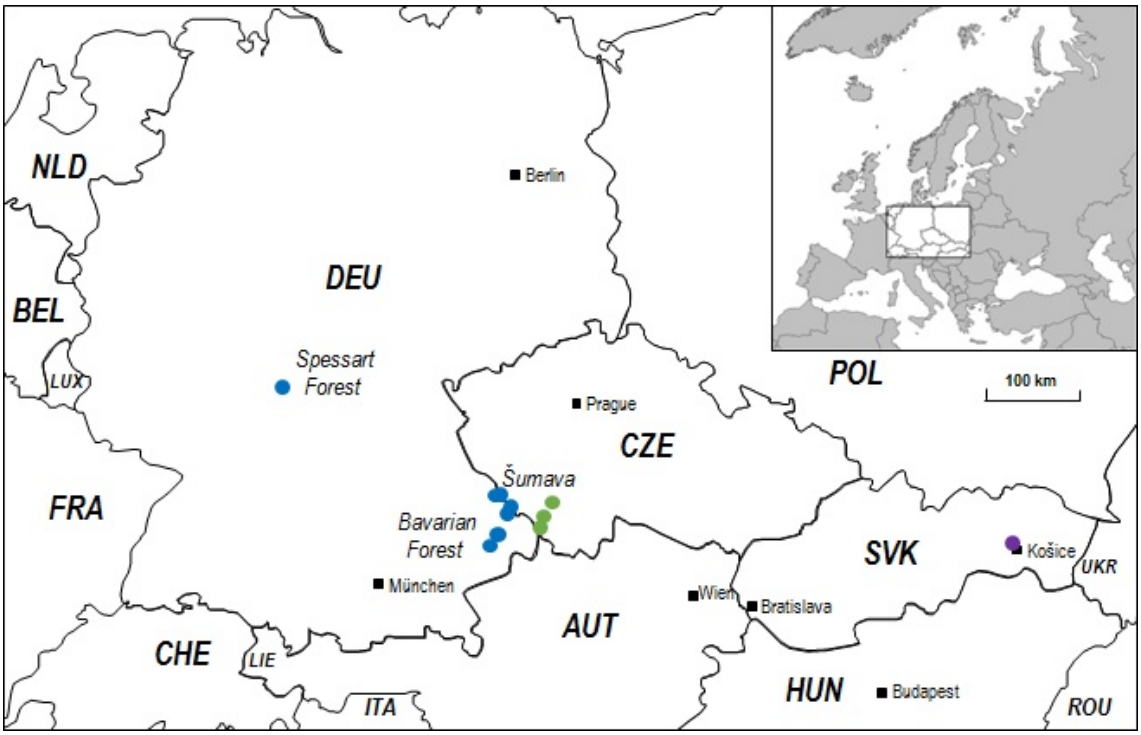

Figure 11: Map of trapping areas with PUUV positive voles in Czech Republic (green dots), Slovakia (violet dots), and Germany (blue dots). In the same area of Slovakia was also trapped a DOBV positive *A. agrarius* individual.

Fifty five individuals belonged to family *Muridae*, subfamily *Murinae* (45 of *Apodemus agrarius* and 10 of *Apodemus flavicollis*). All specimens of the screened rodents of the genus *Apodemus* were trapped in three places nearby Košice (Eastern Slovakia) - Botanická záhrada (Botanic garden), Nižné Kapustníky, Čermeľ. Altogether 51 hantavirus PCR-positive bank voles and 1 stripped field mouse were identified.

4.1.2 Sequencing of hantavirus-positive specimens

At first, we identified the exact species of hantaviruses from the RT-PCR-positive specimens according to partial L segment nucleotide sequences by BLAST. Then, we focused on obtaining of the maximal possible lenght of both, S and M segment for further phylogenetic and sequence analyses (Table 5). We created coherent nucleotide and amino-acid alignments for both- PUUV and DOBV. We made four separate alignments: for S and M segment of PUUV and DOBV. For L segment, which represents the fifth one, we fit newly obtained sequences of PUUV and DOBV together.

After aligning the sequences according to homology, we identified identical sequences in analyzed fragment. In S segment alignment of PUUV, the sequence DO_10_018 was identic with DO_10_031, DO_10_051, DO_10_062, DO_10_063, DO_10_077, DO_10_092, DO_100, DO_10_139, DO_10_156. Then, the sequence T2_10_006 was identical with T2_10_007, T2_10_013, T2_10_039, T2_10_041, T2_10_043, T2_10_046, T2_10_048, T2_10_049, T2_10_052, T2_10_060, T2_10_063, T2_10_080, T2_10_086, T2_10_087, T4_10_012, T4_10_019, T4_10_022, T4_10_030, T4_10_041, T4_10_049, T4_10_053, T4_10_057, T4_10_080, T4_10_081, T4_10_179. The sequence AHW26 was identical with AHW4 and AHW40.

Table 5: List of newly obtained sequences of PUUV from Germany.

				Analyzed segment/lenght [nt]		
Protocol number	Host	Locality	Region	L	S	M
DO_10_008	*C. glareolus*	Iggensbach		-	-	576
DO_10_018	*C. glareolus*	Elsenthal		-	501	576
DO_10_024	*C. glareolus*	Elsenthal		-	-	576
DO_10_031	*C. glareolus*	Elsenthal		-	501	576
DO_10_036	*C. glareolus*	Elsenthal	Bavarian	-	501	576
DO_10_051	*C. glareolus*	Elsenthal	Forest	-	501	576
DO_10_062	*C. glareolus*	Elsenthal	(DEU)	-	501	576
DO_10_063	*C. glareolus*	Elsenthal		-	501	576
DO_10_077	*C. glareolus*	Elsenthal		-	501	576
DO_10_092	*C. glareolus*	Elsenthal		-	501	576
DO_10_100	*C. glareolus*	Elsenthal		-	501	576
DO_10_139	*C. glareolus*	Elsenthal		-	501	576
DO_10_156	*C. glareolus*	Elsenthal		-	501	576
T2_10_006	*C. glareolus*	Rachel		-	501	576
T2_10_007	*C. glareolus*	Rachel		-	501	576
T2_10_013	*C. glareolus*	Rachel		-	501	576
T2_10_039	*C. glareolus*	Rachel		-	501	576
T2_10_041	*C. glareolus*	Rachel		-	501	576
T2_10_043	*C. glareolus*	Rachel		-	501	576
T2_10_046	*C. glareolus*	Rachel		-	501	576
T2_10_048	*C. glareolus*	Rachel	Bavarian	-	501	576
T2_10_049	*C. glareolus*	Rachel	Forest	-	501	576
T2_10_052	*C. glareolus*	Rachel	(DEU)	-	501	576
T2_10_060	*C. glareolus*	Rachel		-	501	-
T2_10_063	*C. glareolus*	Rachel		-	501	576
T2_10_076	*C. glareolus*	Rachel		-	501	576
T2_10_080	*C. glareolus*	Rachel		-	501	576
T2_10_086	*C. glareolus*	Rachel		-	501	576
T2_10_087	*C. glareolus*	Rachel		-	501	576
T2_10_103	*C. glareolus*	Rachel		-	-	576

Table 5: continuation List of newly obtained sequences of PUUV from Germany.

				Analyzed segment/lenght [nt]		
Protocol number	Host	Locality	Region	L	S	M
T4_10_006	*C. glareolus*	Lackenberg		-	501	576
T4_10_012	*C. glareolus*	Lackenberg		-	501	576
T4_10_014	*C. glareolus*	Lackenberg		-	501	576
T4_10_016	*C. glareolus*	Lackenberg		-	-	576
T4_10_019	*C. glareolus*	Lackenberg		-	501	576
T4_10_020	*C. glareolus*	Lackenberg		-	501	576
T4_10_022	*C. glareolus*	Lackenberg		-	501	576
T4_10_026	*C. glareolus*	Lackenberg		-	501	576
T4_10_030	*C. glareolus*	Lackenberg		-	501	576
T4_10_032	*C. glareolus*	Lackenberg		-	501	576
T4_10_041	*C. glareolus*	Lackenberg	Bavarian	-	501	576
T4_10_043	*C. glareolus*	Lackenberg	Forest	-	-	576
T4_10_044	*C. glareolus*	Lackenberg	(DEU)	-	501	576
T4_10_049	*C. glareolus*	Lackenberg		-	501	576
T4_10_053	*C. glareolus*	Lackenberg		-	501	576
T4_10_057	*C. glareolus*	Lackenberg		-	501	576
T4_10_080	*C. glareolus*	Lackenberg		-	501	576
T4_10_081	*C. glareolus*	Lackenberg		-	501	576
T4_10_087	*C. glareolus*	Lackenberg		-	501	576
T4_10_118	*C. glareolus*	Lackenberg		-	501	576
T4_10_127	*C. glareolus*	Lackenberg		-	501	576
T4_10_156	*C. glareolus*	Lackenberg		-	-	576
T4_10_179	*C. glareolus*	Lackenberg		-	-	-
T4_10_203	*C. glareolus*	Lackenberg		-	-	576
AHW26	*C. glareolus*	Wiesenfeld		-	501	-
AHW4	*C. glareolus*	Wiesenfeld	Spessart	-	501	-
AHW6	*C. glareolus*	Wiesenfeld	Forest	-	501	-
AHW40	*C. glareolus*	Wiesenfeld	(DEU)	-	501	-
AHW10	*C. glareolus*	Wiesenfeld		-	501	-

Among the sequences of PUUV M segment alignment, we found a couple of identic sequences in analyzed fragment. The sequence DO_10_018 was identical with DO_10_031, DO_10_036, DO_10_51, DO_10_62, DO_10_63, DO_10_77, DO_10_92, DO_10_100, DO_10_139, DO_10_156 and T2_10_006 was identical with T2_10_007, T2_10_039, T2_10_041, T2_10_043, T2_10_052, T2_10_080, T2_10_086, T4_10_016, T4_10_053, T4_10_057, T4_10_118, T4_10_156. We eliminated all the mentioned identic sequences from the alignments for next analyses.

Table 5: continuation List of newly obtained sequences of PUUV from Slovakia and Czech Republic and of DOBV from Slovakia.

				Analyzed segment/lenght [nt]		
Protocol number	Host	Locality	Region	L	S	M
CZ282/Cg	*C. glareolus*	Žíchovec		226	501	576
CZ286/Cg	*C. glareolus*	Žíchovec		226	501	576
CZ288/Cg	*C. glareolus*	Žíchovec	Bohmerwald	226	501	576
CZ287/Cg	*C. glareolus*	Jelení vrch	(CZE)	226	501	576
CZ6/Cg	*C. glareolus*	Prachatice		226	501	576
CZ7/Cg	*C. glareolus*	Prachatice		226	501	576
SVK129/Cg	*C. glareolus*	Košice-Čermeľ	East	226	501	576
SVK132/Cg	*C. glareolus*	Košice-Čermeľ	Slovakia	226	501	576
SVK780/Cg	*C. glareolus*	Košice-Čermeľ	(SVK)	226	501	576
SVK769/Aa	*A. agrarius*	Košice-Čermeľ		226	519	456

4.1.3 Selecting the Best-Fit model of nucleotide substitutions

The best-fit model of nucleotide substitutions was calculated from these following models: General Time Reversible, Hasegawa-Kishino-Yano, Tamura-Nei, Tamura 3-parameter, Kimura 2-parameter, and Jukes-Cantor. For each model was also calculated additive parameter "Gamma distribution", which assumed that the positions within the alignment could have been evolved by the different speed of evolution (Table 8).

Table 8: Results of model test according to Bayesian Information Criterion (BIC) performed in MEGA7. The best substitution models are those with the lowest values of BIC (highlighted in bold font).

	PUU/DOB L seg.	PUUV S seg.	PUUV M seg.	DOBV S seg.	DOBV M seg.
Model	**BIC**	**BIC**	**BIC**	**BIC**	**BIC**
T92+G+I	**16951,30**	38268,98	17342,48	**26343,96**	**15876,67**
T92+G	16958,77	38284,34	17364,86	26350,48	15899,40
GTR+G+I	16988,82	**38225,07**	17388,90	26367,47	15920,62
GTR+G	16997,48	38276,72	17404,65	26386,05	15937,23
TN93+G+I	16998,48	38297,86	**17341,77**	26354,94	15899,14
HKY+G+I	17021,31	38331,81	17383,47	26389,44	15900,30
HKY+G	17023,71	38355,71	17405,41	26396,49	15924,25
TN93+G	17034,46	38308,15	17384,22	26364,21	15913,72
K2+G+I	17176,20	38473,31	17611,13	26389,24	16022,36
K2+G	17190,02	38517,06	17645,87	26397,91	16050,21
GTR+I	17887,59	40579,95	17851,36	27131,29	16318,49
K2+I	17898,11	40779,30	18027,80	27186,57	16386,75
T92+I	17915,04	40573,54	17875,67	27148,65	16268,10
TN93+I	17932,85	40575,77	17903,50	27158,18	16313,27
HKY+I	18013,37	40677,54	17915,74	27199,99	16291,55
JC+G+I	18063,95	41435,69	18639,10	28256,21	17375,37
JC+G	18073,71	41465,09	18642,16	28270,37	17407,97
JC+I	18700,85	43514,16	18957,98	28900,91	17643,49
GTR	18946,35	43965,82	19801,99	29096,59	17840,50
TN93	18955,75	43980,27	19859,35	29090,79	17851,34
T92	18964,07	43960,92	19851,46	29085,50	17838,67
K2	18977,74	44126,09	19875,08	29121,42	17900,14
HKY	19038,92	44136,96	19902,92	29146,03	17866,11
JC	19738,69	46765,04	20736,81	30740,68	19061,85

Model name abbreviations: GTR: General Time Reversible; HKY: Hasegawa-Kishino-Yano; TN93: Tamura-Nei; T92: Tamura 3-parameter; K2: Kimura 2-parameter; JC: Jukes-Cantor; +G: Gamma dsitribution; +I: Invariable sites.

Then, for each possible model was also calculated another more complex model "Invariable sites", which assumed that some positions within the alignment were not changed during the evolution at all. Models resulted with the lowest values of BIC score were considered as the best. All model tests were performed in MEGA7 (Nei and Kumar, 2000; Kumar *et al.*, 2016).

4.1.4 Phylogenetic analyses

For the best estimation of phylogeny of newly obtained hantavirus genome sequences, we used nucleotide alignments for both, PUUV and DOBV for S and M genome segments separately. The alignment for L segment included PUUV and DOBV sequences together. As an outgroup for L segment phylogenetic tree, we used sequences of *Arvicolinae*- associated hantaviruses (TULV, PHV, ADLV, TATV, Hantavirus YN05-7), *Murinae*- associated hantaviruses (HTNV, SANGV, SEOV), and *Sigmodontinae*- associated hantaviruses (ANDV, SNV, LANV) (Figure 12). The outgroup od PUUV S segment alignment included *Sigmodontinae*- associated hantaviruses (HOKV, MUJV, which are considered as PUUV-like viruses), and *Arvicolinae*- associated hantaviruses (TOPV, KHAV, VLAV, Fusong v., Yuanijang v., Shenyang v.) (Figure 13). The outgroup of PUUV M segment alignment contained the same species of hantaviruses as the PUUV S segment alignment with Yakeshi v. in addition, which represents the group of KHAV-like virus (Figure 14). Ingroup of S and M segment PUUV trees was created from all newly obtained PUUV nucleotide sequences and all PUUV sequences avalaible online in GenBank which fitted into the alignment. Both, the S and M segment of DOBV alignments contained four species of *Murinae*- associated hantaviruses (SANGV, ANJOV, SEOV, HTNV) as the outgroup (Figures 15, 16). Ingroup of S and M segment DOBV trees was made of newly obtained DOBV nucleotide sequences and DOBV sequences of 4 genotypes: Dobrava, Kurkino, Sochi, and Saaremaa, avalaible online in GenBank. Full list of used sequences from GenBank within alignments is available in Appendix 2.

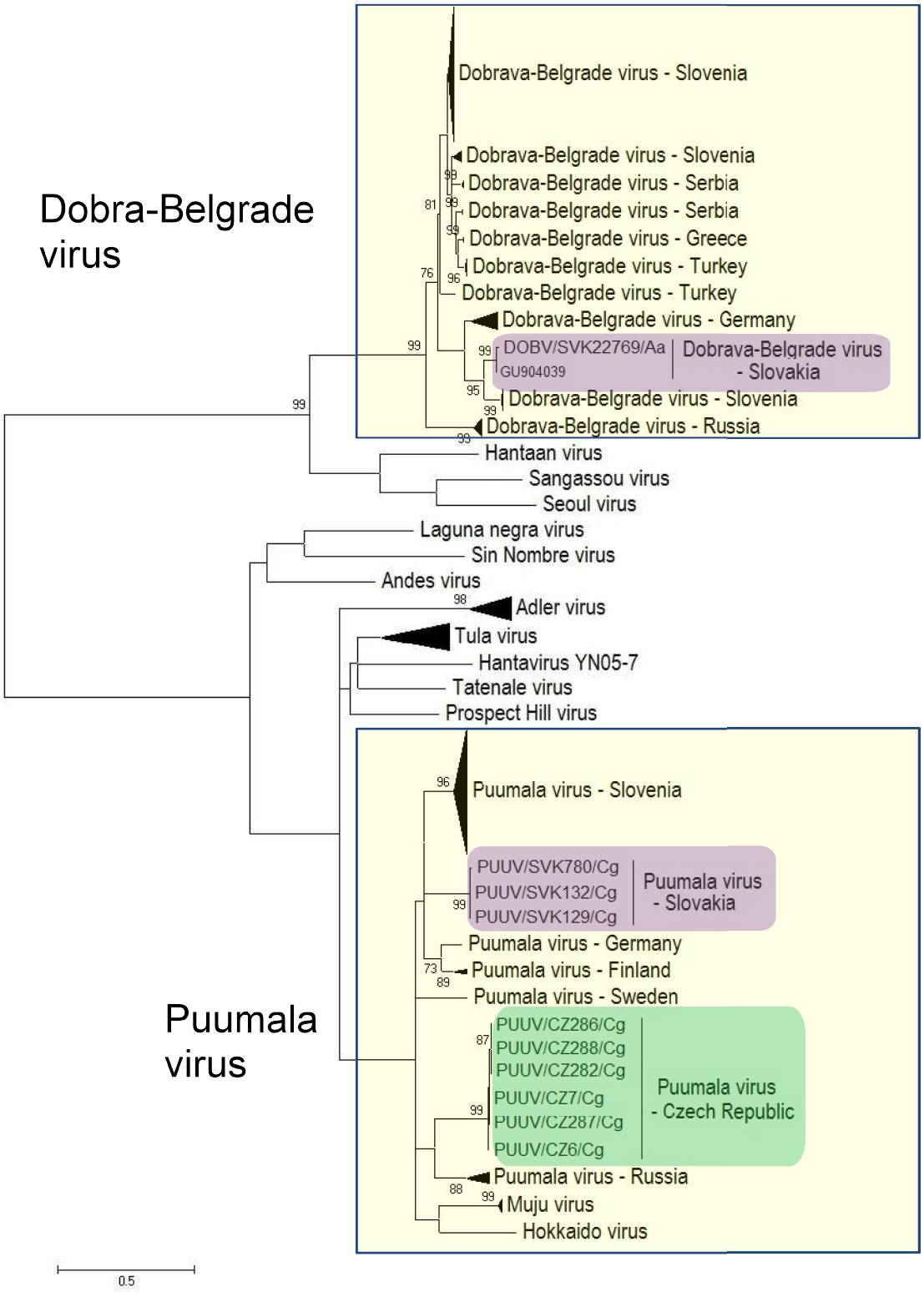

Figure 12: Maximum Lieklihood (ML) phylogenetic tree showing the phylogenetic position of newly detected PUUV strains from Slovakia and Czech Republic and DOBV from Slovakia based on L segment partial sequences (226 nt) made from 229 sequences. More detailed description is available in Appendix 3.

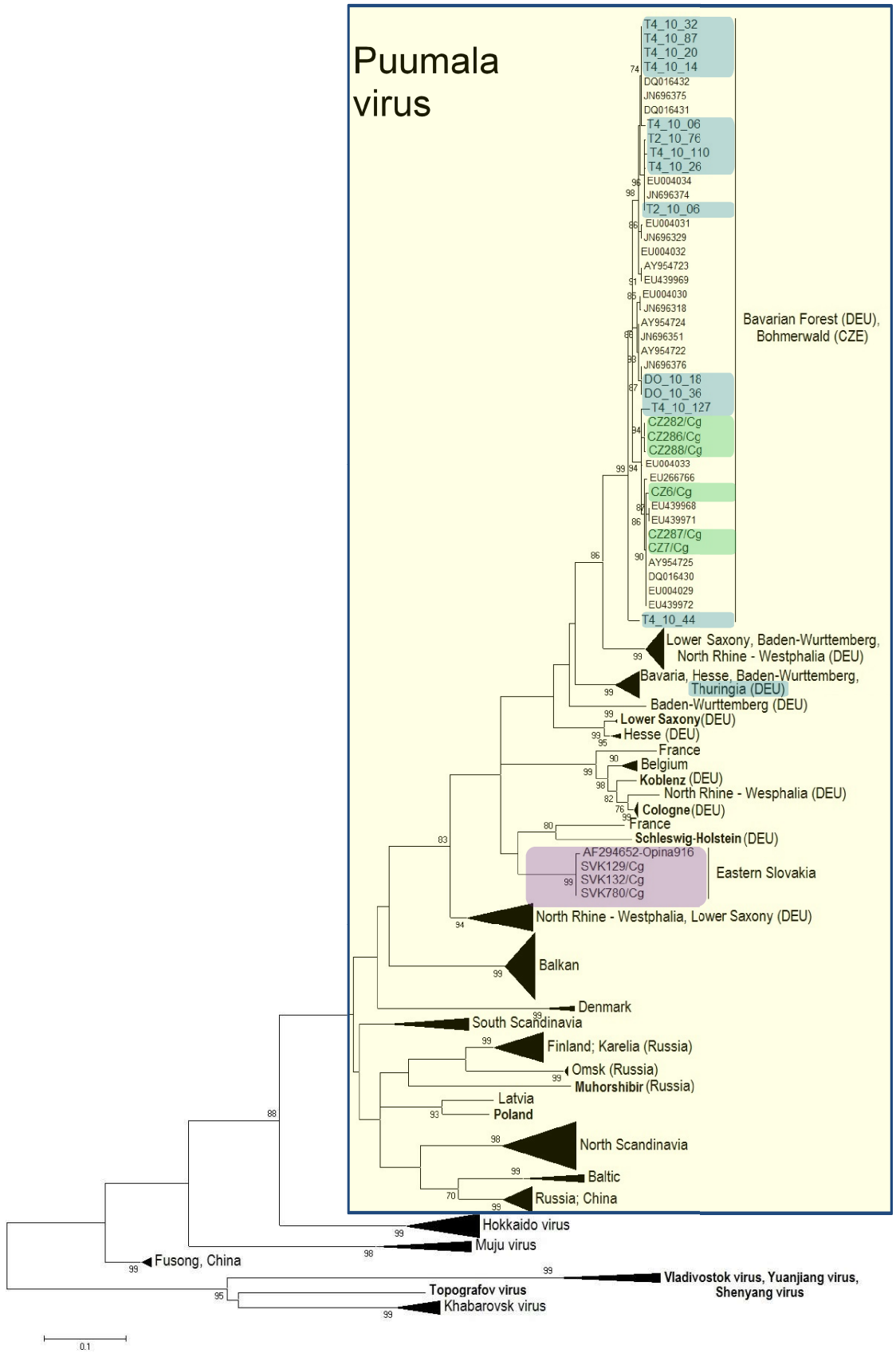

Figure 13: Maximum Lieklihood (ML) phylogenetic tree showing the phylogenetic position of newly detected PUUV strains from Slovakia, Czech Republic and Germany based on S segment partial sequences (501 nt) made from 324 sequences. More detailed description is available in Appendix 3.

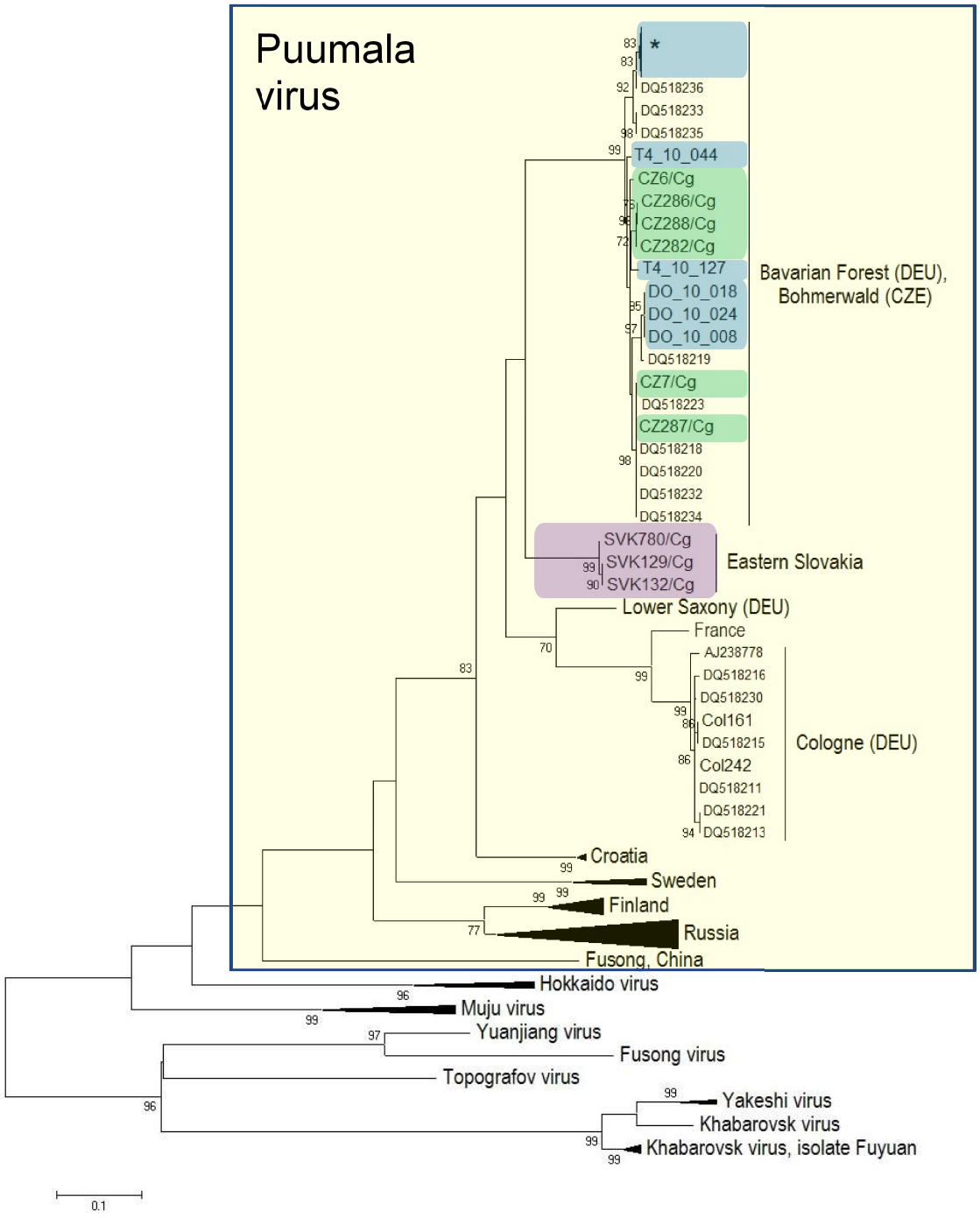

Figure 14: Maximum Lieklihood (ML) phylogenetic tree showing the phylogenetic position of newly detected PUUV strains from Slovakia, Czech Republic and Germany based on M segment partial sequences (576 nt) made from 104 sequences. More detailed description is available in Appendix 3.

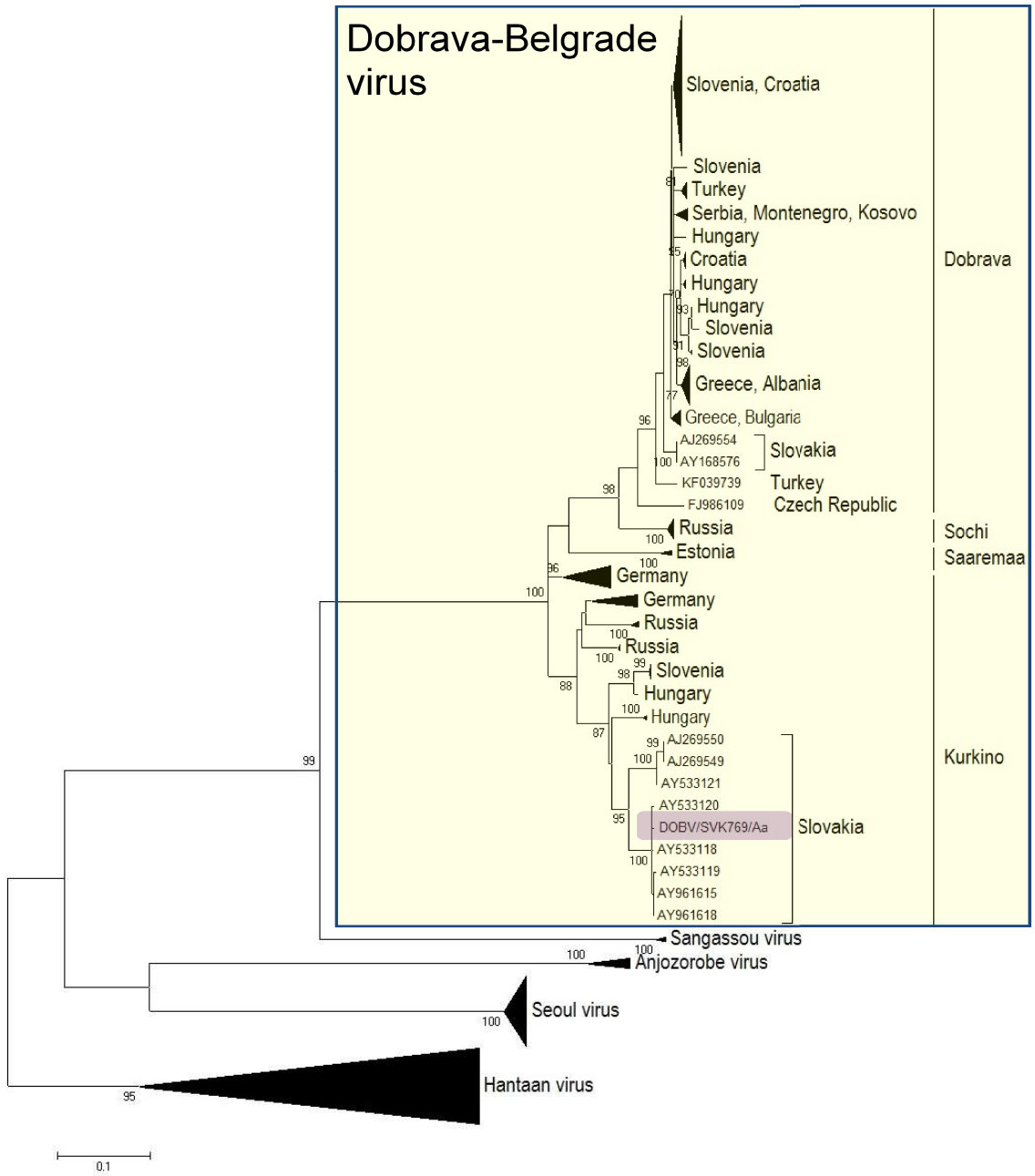

Figure 15: Maximum Lieklihood (ML) phylogenetic tree showing the phylogenetic position of newly detected DOBV strain from Slovakia based on S segment partial sequences (519 nt) made from 268 sequences. More detailed description is available in Appendix 3.

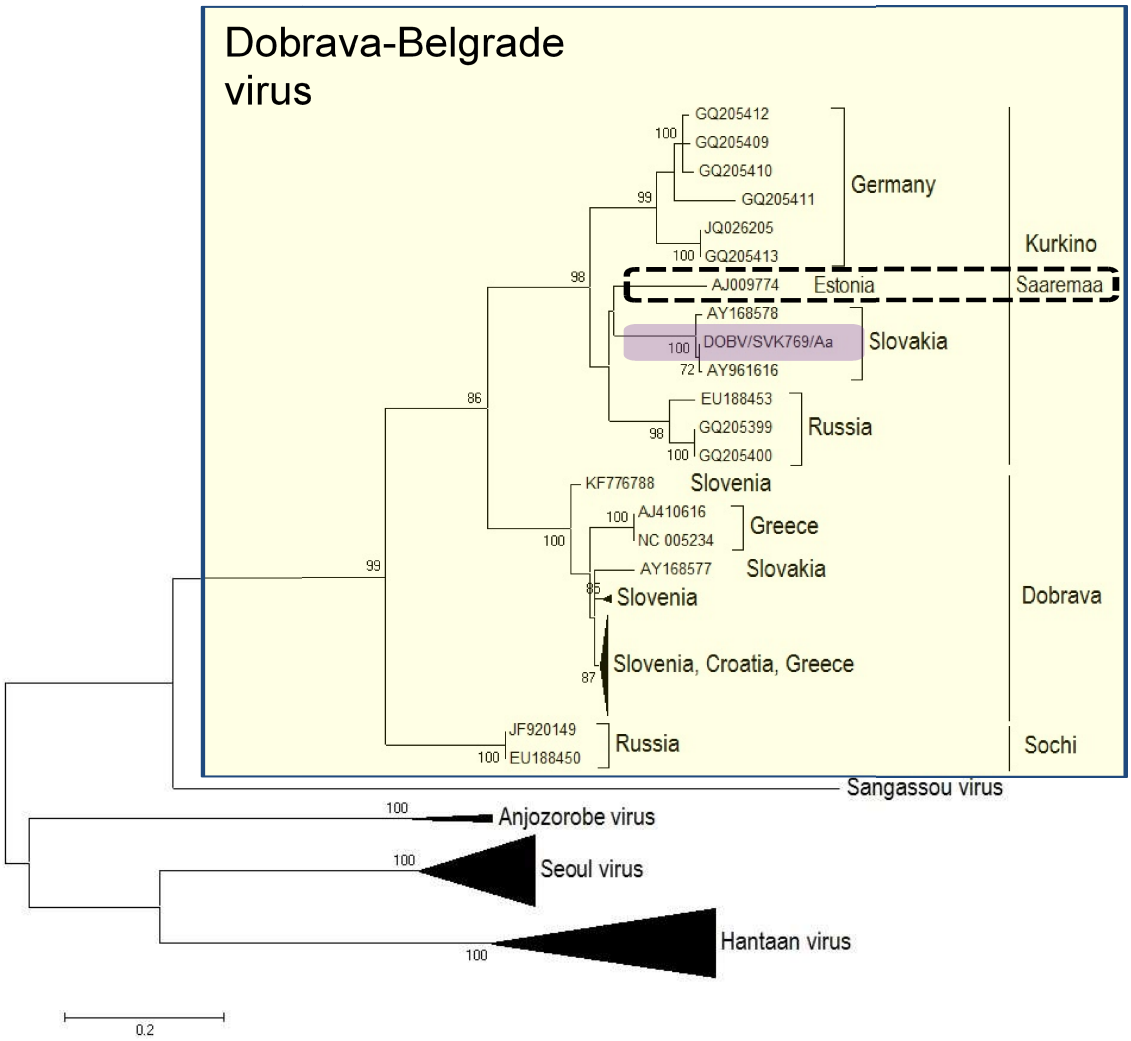

Figure 16: Maximum Lieklihood (ML) phylogenetic tree showing the phylogenetic position of newly detected DOBV strain from Slovakia based on M segment partial sequences (456 nt) made from 127 sequences. More detailed description is available in Appendix 3.

4.1.5 Sequence analyses of PUUV and DOBV genome segments

Analyses based on nt and aa sequence identity matrix performed in Bio Edit support the phylogenetic estimations of our samples. Among PUUV specimens, the highest similarity has been shown between the czech and german samples, 98.4 – 100 % nt and 100 % aa of S-segment and 98.2 – 100 % nt and 99.4 – 100 % aa of M-segment. In contrary, newly obtained slovak sequences showed higher diversity with german samples from Bavaria, 84.6 - 87.0 % nt and 94.6 - 97.0 % aa of S-segment and 86.8 - 88.0 % nt and 96.3 - 97.9 aa of M-segment. In addition, slovak sequences are less similiar to the PUUV and PUUV-like sequences from Finland, Sweden, Japan and South Korea in contrary with czech and german sequences on the nt level (Table 7).

Table 7: Nucleotide (nt) and amino acid (aa) sequence identity analysis of newly obtained PUUV sequences from Eastern Slovakia (SVK), Šumava- Czech Republic (CZE) and Bavaria-Germany (DEU), Numbers express the range of percentual identity between the vertical columns and horizontal rows. Lenght of analyzed sequences: S-segment:501 nt, 167 aa; M-segment: 576 nt, 192 aa.

		SVK		CZE		DEU	
		nt	aa	nt	aa	nt	aa
S segment	SVK	99.8 - 100	99.4 - 100	86.0 - 86.4	96.4 - 97.0	84.6 - 87	94.6 - 97.0
	CZE	86.0 - 86.4	96.4 - 97.0	98.4 - 100	100	95.4 - 98.2	97.0 - 100
	DEU	84.6 - 87.0	94.6 - 97.0	95.4 - 98.2	97.0 - 100	95.4 - 100	95.8 - 100
	Others*	81.8 - 82.0	92.2 - 96.4	76.4 - 81.2	94.0 - 97.0	76.2 - 81.8	92.2 - 97.0
Msegment	SVK	99.6 - 100	99.4 - 100	87.5 - 88.0	96.8 - 97.9	86.8 - 88.0	96.3 - 97.9
	CZE	87.5 - 88.0	96.8 - 97.9	98.2 - 100	99.4 - 100	96.8 - 98.4	98.4 - 100
	DEU	86.8 - 88.0	96.3 - 97.9	96.8 - 98.4	98.4 - 100	96.1 - 100	98.4 - 100
	Others*	77.2 - 82.4	92.1 - 95.3	76.3 - 84.0	92.1 - 96.3	76.5 - 84.3	91.1 - 96.3

Others*(strains available in Gen Bank: PUUV/Sotkamo 2009/FIN; PUUV/Umea/hu, SWE; HOKV/Kitahiyama 123, JPN; MUJV/11-5/KOR).

Sequence identity matrix analysis of DOBV/22769/Aa was performed in comparison with two strains of Dobrava genotype: Slo/Af-BER from Slovenia and 400Af/98 from Eastern Slovakia, then with Kurkino genotype: Lip2/hu (Russia), Saaremaa genotype: 160V (Russia), and Sochi genotype: Ap1584 (Russia). SANGV-SA14 was chosen as the most closest outgroup of DOBV and HTNV-ROKA14-11 as the most distanced outgroup used for phylogenetic analyses (Table 8).

Table 8: Nucleotide (nt) and amino acid (aa) sequence identity analysis of newly obtained DOBV sequences (strain: DOBV/SVK769/Aa) of S and M segment in comparison with representants of four basic genotypes within the DOBV (Dobrava, Kurkino, Saaremaa, and Sochi). Numbers express the range of percentual identity between the vertical columns and horizontal rows. Lenght of analyzed sequences: S-segment:520 nt, 173 aa; M- segment: 456 nt, 151 aa.

		SVK769/Aa		Lip2/hu		400Af/98		Slo/Af-BER		SAAV/160V		Ap1584		SANGV		HTNV	
		nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa
S segment	SVK769/Aa	100	100	88.3	92.3	86	91.1	85	90.5	84.8	88.2	84.8	88.2	75.1	76.6	72.1	69.3
	Lip2/hu	88.3	92.3	100	100	86	88.8	86.8	88.8	86.4	86.3	85.4	86.4	77.5	77.7	71.7	68.7
	400Af/98	86	91.1	86	88.8	100	100	96.5	97	87.4	90.5	91.2	93.5	78.71	76.6	70	67.6
	Slo/Af-BER	85	90.5	86.8	88.8	96.5	97	100	100	87.4	59.4	91.2	92.9	78.6	76.6	69.8	65.8
	SAAV/160V	84.8	88.2	86.4	86.3	87.4	90.5	87.4	89.4	100	100	85.4	86.9	76.5	77.7	71.9	67
	Ap1584	84.8	88.2	85.4	86.4	91.2	93.5	91.2	92.9	85.4	86.9	100	100	77.3	77.7	69.6	68
	SANGV	75.1	76.6	77.5	77.7	78.1	76.6	78.6	76.6	76.5	77.7	77.3	77.7	100	100	69.4	68.7
	HTNV	72.1	69.3	71.7	68.7	70	67.6	69.8	65.8	71.9	67	69.6	68	69.4	68.7	100	100
M segment	SVK769/Aa	100	100	87.9	98	82.6	96	81.7	96	87	98.6	78.9	93.3	72.5	84.1	71.2	79.4
	Lip2/hu	87.9	98	100	100	83.3	96	83.7	96	85.7	98	81.3	92	71.9	83.4	73	78.8
	400Af/98	82.6	96	83.3	96	100	100	94.7	100	81.5	97.3	81.1	95.3	73.2	85.4	71.2	80.1
	Slo/Af-BER	81.7	96	83.7	96	94.7	100	100	100	81.1	97.3	80.7	95.3	72.5	85.4	71.7	80.1
	SAAV/160V	87	98.6	85.7	98	81.5	97.3	81.1	97.3	100	100	79.1	94	73.2	84.7	71	78.8
	Ap1584	78.9	93.3	81.3	92	81.1	95.3	80.7	95.3	79.1	94	100	100	71.7	83.4	71	80.1
	SANGV	72.5	84.1	71.9	83.4	73.2	85.4	72.5	85.4	73.2	84.7	71.7	83.4	100	100	69.7	79.4
	HTNV	71.2	79.4	73	78.8	71.2	80.1	71.7	80.1	71	78.8	71	80.1	69.7	79.4	100	100

Dobrava: Slo/Af-BER (Slovenia); Dobrava: 400Af/98 (Slovakia); Kurkino: Lip2/hu (Russia), Saaremaa: 160/V (Russia); Sochi: Ap1584; HTNV: ROKA14-11; SANGV: SA14.

Our newly obtained specimen of DOBV (22769/Aa) was mostly similar with Kurkino genotype, strain Lip2/hu in both, S and M segment comparisons (88.3 % nt and 92.3 % aa of S-segment and 87.9 % nt and 98 % aa of M-segment). As the second most similar was Dobrava genotype, strain 400Af/98 from Eastern Slovakia (86 % nt and 91.1 % aa) in S segment, but in M segment the second mostly similar was Saaremaa genotype, strain 160V (87 % nt and 98.6% aa). SANGV, strain SA14, as the closest outgroup of DOBV resulted with higher sequence similarity (75.1 % nt and 76.6% in aa in S segment, and 72.5 % nt and 84.1 % in M segment) than HTNV, strain ROKA14-11 (72.1 % nt and 69.3 % aa in S segment, and 71.2 % nt and 79.4 % in M segment). Obviously, the percentage results of identity matrix analyses correspond to the length of branches and the topology of phylogenetic trees. It is notable, that the molecular evolution of S and M segment was not parallel, even though we analysed only partial segments. The analyses of both, PUUV and DOBV, S and M sequences suggest, that there is a higher probability for a reassortment in M segment than in S segment due to significantly higher percentual differences in M segment sequences.

4.1.6 Virus isolation attempts

RT-PCR-positive lung tissue samples from wild bank voles trapped in East Slovakia (Cat. No.: PUUV/SVK129/Cg, PUUV/SVK132/Cg, PUUV/SVK780/Cg) were seeded onto VERO E6 cell cultures for the isloation of infectious PUUV. After 4 passages (8 weeks), we extracted the RNA from cell cultures and media of all passages with TRIzol® Reagent (Thermo Fisher Scientific, MA, USA), and screened by RT-PCR assay (Klempa *et al.*, 2006).

Based on the RT-PCR screening, beacuse of that all the cell passages (cells and media) were hantavirus-negative, we did not continue with immunofluorescence assay as we had planned at the start. In conclusion, the virus isolation experiment was unsuccessfull. PUUV-positive tissue samples of bank voles obtained from the Czech Republic from bank voles captured in 2007 and 2008 (Cat. No.: PUUV/CZ6/Cg, PUUV/CZ7/Cg, PUUV/CZ282/Cg, PUUV/CZ286/Cg, PUUV/CZ287/Cg, and PUUV/288/Cg) were not chosen for the virus isolation experiments due to the lack of the amount of the infectious tissue samples which were almost all used for the

molecular biological experiments. In fact, from two of them (Cat. No.: PUUV/CZ282/Cg, PUUV/CZ286/Cg) we have had only the RNA solution available.

Isolation of hantaviruses is a process taking a long time and it is only rarely successful. For example, when DOBV strain "Slo/Af" was isolated, only one out of 13 isolation experiments finished successfully (Avsic-Zupanc *et al.*, 1992). We assume that a possible reason of the failure could been also a too long time taken to store our tissue samples (since 2012) and the fact, that they have been melted and freezed again several times for the reason of molecular biologic experiments to obtain genome sequences data. Even though the infectiousness could have been lost, the virus genome fragments were still detectable for molecular analyses.

4.2 Bacteriophage MS2 as an internal control in duplex RT-PCR assay for the screening of hantaviruses

We used a part of original protocol of duplex RT-PCR assay with bacteriophage MS2 as exogenous IC originally developed for the purpose of targeting hepatitis C virus RNA in blood donor screening (Dreier *et al.*, 2005). This assay was originally designed as the PCR assay of only one round of cycles, so we modified it with a novel second pair of primers annealing on the phage MS2 RNA: "MS2:2781F" and "MS2: 2989R" for the nested RT-PCR and implemented them into the nested RT-PCR assay also called as Pan-Hanta-L-PCR assay for the general screening of different species of hantaviruses (Klempa *et al.*, 2006).

Different concentrations of infectious control solution containing Prospect Hill virus (PHV) were tested (1×10^3, 1×10^2, 1×10^1 and 1 FFU/ml). 1×10^3 FFU/ml resulted as still detectable. More diluted virus inoculums resulted as hantavirus-negative. As we added 50 µl of the PHV inoculum, the tissue contained 20 virus particles. Based on our results, obviously the best option for the duplex RT-PCR is the combination of primers of Pan-Hanta-PCR assay in use as they were originally designed with primers for the bacteriophage MS2 in the second round of RT-PCR: 2781F/3031R (semi-nested) or 2781F/2989R (nested) (Figure 17). In conclusion, we demonstrated the original Pan-Hanta-PCR assay using the bacteriophage MS2 as an internal genomic control. The results confirm a wide usefulness of the MS2 phage as a non-competitive

internal control for different assays. Moreover, duplex RT-PCR assy could be a helpful additional tool in combination with serologic tests, such as IgM/IgG ELISAs in clinical diagnostics of hantavirus diseases.

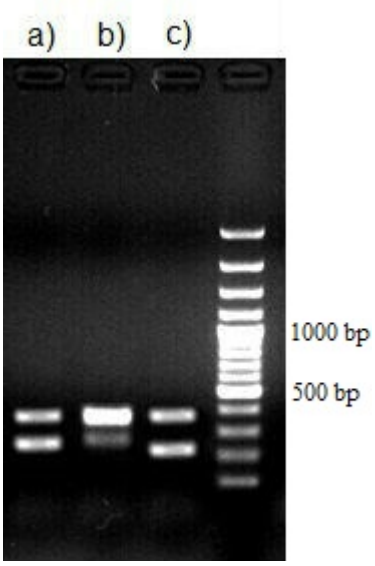

Figure 17: [a)] MS2: 2781F / MS2: 3031R [b)] MS2: 2717F / MS2: 2989R [c)] MS2: 2781F / MS2: 2989R. Product size: [a)] 250 bp [b)] 272 bp [c)] 208 bp; Upper band in the all three runs is a product of primer combination: HANL-F2/HANLR2, product size: 390 bp; 50 µl of PHV inoculum was added into the each sample with the titer 1 x 10^3 FFU/ml; Ladder: GeneRuler 100 bp DNA Ladder (Thermo Scientific, MA, USA).

4.3 Screening of biological activity of selected chemical compounds

4.3.1 Antiviral activity of aryl-piperazine derivates

A novel series of seventeen aryl-piperazine derivates were evaluated for their antiviral activity. All derivates were screened primarily against hantaviruses (PUUV and SEOV) and Yellow fever virus (YFV), as representatives from the genera *Orthohantavirus* (family: *Hantaviridae*) and *Flavivirus* (family: *Flaviviridae*), causing hemorrhagic fevers. Next, these compounds were evaluated against HIV-1 (family: *Retroviridae*), Coxsackie virus B5 (family: *Picornaviridae*), Vesicular stomatitis virus (family: *Rhabdoviridae*) and Vaccinia virus (family: *Poxviridae*). The rationale of testing aryl-piperazine derivates against the latter viruses, which are representatives of families mentioned in parenthesis, was to gain useful information on the importance of the polarity and/or segmentation of the viral genome for the antiviral activity of the inhibitors.

The 6-azauridine (AzUR, 6-aza), which was used as a title compound besides of Ribavirin for the antiviral evaluation of hantaviruses, showed approximately two times lower concentration needed for 50% inhibition of the replication of both, PUUV and SEOV (6-aza: EC_{50} = 5 μM; RBV: EC_{50} = 21 μM). Interestingly, 6-aza as an inhibitor of hantavirus replication, has not been yet published. Among the aryl-piperazine derivates, none of them showed activity against hantaviruses while some of them resulted as cytotoxic against VERO E6 (CC_{50} = 10 - 20 μM) (Tables 9, 10). The experiments on hantaviruses were perfomed by focus-reduction assay visualized by chemiluminescence.

The antiviral activity of aryl-piperazine derivates against both, Yellow fever virus (YFV) cultivated on BHK-21 cells and Human immunodeficiency virus (HIV-1) on MT-4 cells, was evaluated by MTT test. In both cases, no antiviral activity was observed. The Efavirenz, which was used as a title inhibitor of HIV-1, although showed moderate cytotoxicity against MT-4 cells (CC_{50} = 38 μM), confirmed its highly selective activity against HIV-1 (EC_{50} = 0.0026 μM). The 2'-β-methylguanosine (2'-C-ME-Guo, NM 108) was used as a title compound for YFV. Against BHK-21 cells, we observed no cytotoxicity (CC_{50} >100 μM), and inhibitory effect on YFV in the low micromolar concentrations (EC_{50} = 1.87 μM). None of the aryl-piperazines derivates inhibited the

replication of YFV and twelve of them resulted cytotoxic against BHK-21 (Tables 9, 10).

Some of the aryl-piperazine derivates (2Cl3C2, 2Cl3C6, 2Cl3C10, 2Cl4C5, 2Cl4C6, 2Cl4C7, 2Cl4C8, 2Cl4C12) significantly showed an antiviral activity against the Coxsackie virus B5 (CVB-5). The EC_{50} was determined by the plaque reduction assay (EC_{50} range = 4.4-18 μM). The EC_{50} of the title compound Pleconaril was 0.005 μM and it showed no cytotoxicity against the VERO76 cells (CC_{50}>100 μM) (Table 16 and 17). Plaque reduction assay was used also for Vaccinia virus (VV) and Vesicular stomatitis virus (VSV) vultivated on VERO76. Against the Vaccinia virus, none of the aryl-piperazine derivates were active while the title compound Mycophenolic acid showed a significant inhibition with EC_{50} = 2 μM. No antiviral activity was shown against the VSV as well.

Structure-activity relationship (SAR) studies indicate that the structure and conformation of the terminal aryl- (or phenyl-) group and the lenght of the linear hydrocarbon chain binding the aryl-piperazine group could be responsible for the antiviral effect of the aryl-piperazine derivates. There were two forms of linear hydrocarbon chain present: propyl- group **2** (3 carbons) or butyl- group **3** (4 carbons).

The compound 2Cl3C2 showed an antiviral activity against CVB-5 with EC_{50} = = 18 μM. Most likely, the presence and conformation of the chlorine within the phenyl group (4-chlorophenyl- group, **2b**) played a role in its antiviral activity. On the other hands, the absence of antiviral activity of the compound 2Cl3C13 which contained the same molecule (i.e. 4-chlorophenyl piperazine), suggests that the lost of activity might be caused by the presence of one more molecule of the cyclic hydrocarbon chain bound (4-chlorophenyl-phenyl-methyl-piperazine, **2l**).

The 2Cl3C5 showed the EC_{50} = 18 μM, but associated with high cytotoxicity (CC_{50} = 30 μM) against VERO 76 host cells. Interestingly, the similar chemical compound 2Cl4C5, which differs only by one atom of carbon more in its linear hydrocarbon chain binding the whole aryl-piperazine group, **3e** (a butyl- group, **3e**, instead of a propyl- group, **2e**, as in 2Cl3C5), showed a significant antiviral effect against CVB-5 (EC_{50} = 15.8 μM) but no cytotoxicity (CC_{50} >100 μM). On the contrary, compounds 2Cl3C6 and 2Cl4C6, which differ one another for a propyl- and butyl-group, respectively (**2f**, **3f**), showed anti- CVB-5 activity (EC_{50} = 16 and 4.4 μM,

respectively) associated to none to a low cytotoxic effect (CC_{50} >100 and = 50 μM, respectively). According to these results, it is assumable that the presence of pyridine-2yl group (**f**) instead of the pyrazin-2-yl group (**e**) within the aryl-piperazine group is also important, than only the presence of butyl-group hydrocarbon linear chain **2** binding the aryl-piperazine group to the rest of the molecule.

According to the results of 2Cl3C10, we can assume that there could be some importance in antiviral activity against CVB-5, when on the terminal aryl- group of the molecule is bound an atom from the group of halogens on the 4th carbon (4-fluorophenyl, **2j**) of the molecule. This is supported by the results with the 2Cl3C2 compound, which has exactly the same structure and conformation as 2Cl3C10 with the only difference being the substitution of the 4-fluorophenyl group (**2j**) with a 4-chlorophenyl group (**2b**). It is important to note, that both 2Cl3C2 and 2Cl3C10 have the aryl-piperazine group bound to only the 3-hydrocarbon linear chain (propyl-group, **2**). Interestingly, the 2Cl4C10 with the same 4-fluorophenyl group, which differs in its linear carbon chain prolonged only by one atom of the carbon (butyl-group, **3**), resulted as inactive.

The 2Cl4C7 showed a moderate although selective antiviral activity (EC_{50} = 10.5 μM vs. CC_{50} = 50 μM). In this case, the conformation of two nitrogens bound within the phenyl-group (pyrimidin-2-yl, **3g**) could be mainly responsible for the activity. On the other hands, the very similar derivate 2Cl4C5, which differs for a different conformation of the terminal phenyl-group (pyrazin-2yl, **3e**), showed better activity (see above).

Compounds 2Cl3C8 and 2Cl4C8 represent two examples of simple aryl-piperazine derivates in which the terminal phenyl-group is represented only by the cyclic hydrocarbon chain without a substitution by any other atom. The only difference between 2Cl3C8 and 2Cl4C8 reside in their linear hydrocarbon chain binding the aryl-piperazine group (propyl-, **2** and butyl- **3** group, respectively). In this case, 2Cl4C8 showed a promising antiviral acitivity (EC_{50} = 11.8 μM) with no notable cytotoxicity (CC_{50} >100 μM).

It seems that 2-etoxyphenyl group might be also a notable difference when bound in aryl-piperazine derivates, as it showed the activity with the 2Cl4C12 (**3k**) (EC_{50} = 12.5 μM).

Table 9: Cytotoxicity and antiviral activity of aryl-piperazine derivates and reference compounds against representatives of ssRNA$^+$ (HIV-1, YFV, CBV-5), ssRNA$^-$ (VSV, SEOV, PUUV) and dsDNA (VV) viruses.

Compound	MT-4	HIV-1	BHK	YFV	VERO76	CVB-5	VSV	VV	VERO-E6	SEOV	PUUV
	[a]CC_{50}	[b]EC_{50}	[c]CC_{50}	[d]EC_{50}	[e]CC_{50}	[f]EC_{50}	[g]EC_{50}	[h]EC_{50}	[i]CC_{50}	[j]EC_{50}	[k]EC_{50}
2Cl3C1	14	>14	11.86	>11.86	>100	>100	>100	>100	20	>20	>20
2Cl3C2	16	>16	14.64	>14.64	>100	18	>100	>100	15	>15	>15
2Cl3C3	>100	>100	>100	>100	>100	53	>100	>100	>30	>30	>30
2Cl3C5	18	>18	35.9	>35.9	30	18	>30	>30	20	>20	>20
2Cl3C6	>100	>100	>100	>100	>100	16	>100	>100	>30	>30	>30
2Cl3C8	>100	>100	>100	>100	>100	41	>100	>100	>30	>30	>30
2Cl3C9	>100	>100	>100	>100	>100	>100	>100	>100	>30	>30	>30
2Cl3C10	16	>16	13.48	>13.48	50	16.5	>50	>50	15	>15	>15
2Cl3C13	14	>14	13,37	>13.37	>100	>100	>100	>100	10	>10	>10
Ribavirin									>100	21	21
Efavirenz	38	0,0026									
Pleconaril					>100	0.005					
2'-C-Me-Guo			>100	1,87							
6-Azauridine									>100	5	5
Mycophen. Ac.					>100			2			

[a] Compd concn (µM) required to reduce by 50% the proliferation of mock-infected MT-4 cells, as determined by the MTT at 4th day; [b] Compd concn (µM) required to achieve 50% protection of MT-4 cells from HIV-1-induced cytopathogenicity, as determined by the MTT at 4th dpi.; [c] Compd concn (µM) required to reduce by 50% the viability of mock-infected BHK cells, as determined by the MTT at 3rd day; [d] Compd concn (µM) required to achieve 50% protection of BHK cells from YFV$^{(d)}$- induced cytopathogenicity, as determined by the MTT at 3rd dpi.; [e] Compd concn (µM) required to reduce by 50% the viability of mock-infected Vero-76 cells, as determined by the MTT at 3rd/5th day; [f-h] Compd concn (µM) required to reduce by 50% the plaque number of CVB-5$^{(f)}$, VSV$^{(g)}$, VV$^{(h)}$ in Vero-76 cells at 3rd dpi.; [i] Compd concn (µM) capable to compromise the integrity of Vero E6 monolayers, as determined by visual examination at 7th day.; [j] Compd concn (µM) required to reduce by 50% the number of SEOV foci in Vero E6 cells at 7th dpi.; k Compd concn (µM) required to reduce by 50% the number of PUUV foci in Vero E6 cells at 14th dpi.

Table 10: Cytotoxicity and antiviral activity of aryl-piperazine derivates and reference compounds against representatives of $ssRNA^+$ (HIV-1, YFV, CBV-5), $ssRNA^-$ (VSV, SEOV, PUUV) and dsDNA (VV) viruses.

Compound	MT-4	HIV-1	BHK	YFV	VERO76	CVB-5	VSV	VV	VERO-E6	SEOV	PUUV
	[a]CC_{50}	[b]EC_{50}	[c]CC_{50}	[d]EC_{50}	[e]CC_{50}	[f]EC_{50}	[g]EC_{50}	[h]EC_{50}	[i]CC_{50}	[j]EC_{50}	[k]EC_{50}
2Cl4C1	13	>13	7.35	>7.35	>100	>100	>100	>100	10	>10	>10
2Cl4C4	>100	>100	>100	>100	>100	>100	>100	>100	>30	>30	>30
2Cl4C5	30	>30	36.62	>36.62	>100	15.8	>100	>100	>30	>30	>30
2Cl4C6	15	>15	8.94	>8.94	50	4.4	>50	>50	20	>20	>20
2Cl4C7	18	>18	21	>21	50	10.5	>50	>50	20	>20	>20
2Cl4C8	14	>14	8.58	>8.58	>100	11.8	>100	>100	20	>20	>20
2Cl4C10	14	>14	9.76	>9.76	>100	>100	>100	>100	20	>20	>20
2Cl4C12	12	>12	8.14	>8.14	50	12.5	>50	>50	10	>10	>10
Ribavirin									>100	21	21
Efavirenz	38	0,0026									
Pleconaril					>100	0,005					
2'-C-Me-Guo			>100	1,87							
6-Aza-Uridine									>100	5	5
Mycoplen. Ac.					>100			2			

[a] Compd concn (µM) required to reduce by 50% the proliferation of mock-infected MT-4 cells, as determined by the MTT at 4th day; [b] Compd concn (µM) required to achieve 50% protection of MT-4 cells from HIV-1-induced cytopathogenicity, as determined by the MTT at 4th dpi.; [c] Compd concn (µM) required to reduce by 50% the viability of mock-infected BHK cells, as determined by the MTT at 3rd day; [d] Compd concn (µM) required to achieve 50% protection of BHK cells from YFV[(d)]- induced cytopathogenicity, as determined by the MTT at 3rd dpi.; [e] Compd concn (µM) required to reduce by 50% the viability of mock-infected Vero-76 cells, as determined by the MTT at 3rd/5th day; [f-h] Compd concn (µM) required to reduce by 50% the plaque number of CVB-5[(f)], VSV[(g)], VV[(h)] in Vero-76 cells at 3rd dpi.; [i] Compd concn (µM) capable to compromise the integrity of Vero E6 monolayers, as determined by visual examination at 7th day.; [j] Compd concn (µM) required to reduce by 50% the number of SEOV foci in Vero E6 cells at 7th dpi.; k Compd concn (µM) required to reduce by 50% the number of PUUV foci in Vero E6 cells at 14th dpi.

4.3.2 Anti-cancer activity of aryl-piperazine derivates

As reported above, a number of the aryl-piperazine derivates resulted cytotoxic against BHK-21 cells (CC_{50} range = 7.35 – 36.62 μM). Those endowed with the highest cytotoxicity ($CC_{50} \leq 10$ μM) i.e. compounds 2Cl4C1, 2Cl4C6, 2Cl4C8, 2Cl4C10, and 2Cl4C12, were selected for evaluating their antiproliferative activity against human cancer cell lines; in particular cervix (HeLa), skin (SK-MEL-28), lung (SK-MES-1), prostate (DU145), peripheral blood (CCRF-CEM, CCRF-SB), and spleen (WIL-2NS). CRL-7065 cells derived from foreskin of newborn were used as a non-tumoral cell control. All cytotoxicity screening of test compounds against tumoral and non-tumoral cell lines were performed by MTT-test.

Two compounds, 2Cl4C8 and 2Cl4C10, showed a good antiproliferative activity against HeLa, SK-MEL-28, SK-MES-1, CCRF-CEF, CCRF-SB, and WIL-2NS cells (CC_{50} range = 7.65 -15.5 μM), with no notable cytotoxicity against non-tumoral cell control CRL-7065 ($CC_{50} > 100$ μM). Other compounds, 2Cl4C1, 2Cl4C6, and 2Cl4C12, did not show selectivity for cancer cells as they showed comparable cytotoxicity against CRL-7065, too (CC_{50} = 39.37 – 54.19 μM).

It is interesting to note that the derivate 2Cl4C8, which resulted as one of the most potent against CVB-5, resulted also highly cytotoxic against the mentioned cancer cells. Obviously, the butyl-group in combination with the simple terminal phenyl-group, without substitutions within the aryl-piperazine derivate (**3h**), is a structure with promising biological activities. On the other hands, 2Cl4C10 resulted also a promising chemical of the class, as it inhibited the proliferation of tumoral cells. In this case, the butyl-group with the terminal 4-fluorophenyl group of the molecule (**3j**) could likely play a role.

Vincristine was used as a title compound for all tumoral cells. Vincristine was previously reported effective against different types of tumoral cells (Lengsfeld *et al.*, 1981; Couter and Beck, 1984). In my hands, it resulted more potent against human leukemia derived cell lines CCRF-CEM, CCRF-SB, and WIL-2NS (CC_{50} = 0.69 – 8.43 μM) (Table 11).

Table 11: Antiproliferative activity od aryl-piperazine derivates and reference compound against representatives of tumoral cell cultures (HeLa, SK-MEL-28,, SK-MES-1, DU 145, CCRF-CEM, CCRF-SB, WIL-2NS) and non-tumoral control cells (CRL-7065).

Compound	HeLa	SK-MEL-28	SK-MES-1	DU145	CCRF-CEM	CCRF-SB	WIL-2NS	CRL-7065
	[a]CC_{50}	[b]CC_{50}	[c]CC_{50}	[d]CC_{50}	[e]CC_{50}	[f]CC_{50}	[g]CC_{50}	[h]CC_{50}
2Cl4C1	7,9	13,49	6,5	>100	5,88	8,93	6,72	54,19
2Cl4C6	10,4	12	6,3	38,14	7,38	8,49	7,66	44,86
2Cl4C8	10	15,1	8,89	95,7	8,47	10,57	7,65	>100
2Cl4C10	12,67	15,5	9,2	>100	10,57	11,6	7,82	>100
2Cl4C12	11,8	11,2	5,57	68,54	5,04	5,46	7,58	39,37
Vincristine	19,89	36,987	>100	15,64	0,69	1,888	8,43	>100

(a-h) Compd concn (μM) required to reduce by 50% the proliferation of [a]HeLa cells, bSK-MEL-28 cells, cSK-MES-1 cells, [d]DU 145, eCCRF-CEM, fCCRF-SB cells, gWIL-2NS cells and hCRL-7065 cells as determined by the MTT method at day 3 following treatment.

5. DISCUSSION

5.1 Occurence of PUUV in Central Europe

Based on the phylogenetic trees, mainly of S and M segment of PUUV, the newly obtained PUUV-positive specimens from Šumava National Park (Böhmerwald), Czech Republic, are most closely related to the strains from neighbouring Bavarian Forest National Park (BFNP) (100% boostrapping support). Unfortunately, due to a lack of L segment sequence data from bank voles collected in the BFNP, is difficult to confirm the other evolutionary links across all genome segments.

The clustering of L segment tree is statistically poorly supported, which could be most likely caused by the insufficient lenght of the L segment alignment (226 nt). In the S and M segment trees, they are obviously at the origin of the currently circulating Finnish PUUV lineages. The L segment topologies could indicate a reassortment event, but it cannot state it as a conclusion without dubiousness such as it is based on the short-length L segment alignment.

The S segment sequences of the newly identified Slovak strains form a statistically well supported sister clade of the PUUV strains which are circulating among bank voles in Belgium, France, and regions in north-western Germany. But the topology of the M segment alignment is quite different and may refer about the reassortment event or, as only the partial sequences were analyzed, a homolougous recombination, too. Particularly in the M segment, the Slovak strains are clustered in the same clade as the strains from Czech Republic and the BFNP, and then are more closely related to those from France and north-western Germany. The reassortment scenario is inferred more likely such as homologous recombination in hantavirus genome which has been also previously reported (Sibold *et al.*, 1999; Klempa *et al.*, 2003; Nicolic *et al.*, 2014).

Obviously, reassortment seems to be a more widely occurring event among hantaviruses (Henderson *et al.*, 1995; Li *et al.*, 1995; Klempa *et al.*, 2003), PUUV including (Razzauti *et al.*, 2008; Razzauti *et al.*, 2009; Razzauti *et al.*, 2013) what is in contrary to that what had been thought when the rules of the hantavirus species demarcation were established (Plyusnin *et al.*, 2012). The reassortment in hantaviruses

was demonstrated *in vitro* as well (Rizvanov *et al.*, 2004; Kirsanovs *et al.*, 2010; Handke *et al.*, 2010). Interestingly, the indications of reassortment and/or recombination were previously reported from Eastern Slovakia for Tula virus nad Dobrava-Belgrade virus, too (Sibold *et al.*, 1999; Klempa *et al.*, 2003). This phenomenon might reflect that this region has been involved in several end-glacial colonization routes of rodents. According to the mtDNA analyses, Easten Slovakia represents a contact area of three phylogeographic clades of bank voles (the Carpathian, the Western and the Eastern) (Filipi *et al.*, 2015), thus it was a good opportunity for the occurrence of several PUUV lineages.

5.2 Detection of DOBV in Eastern Slovakia

There had been a lot of dubiousnesses about the classification of DOBV species until the four genotypes of DOBV (Dobrava, Kurkino, Sochi and Saaremaa) were described in 2013 and it was declared that Saaremaa virus cannot be more classified as an independent virus species within the genus *Hantavirus* (Klempa *et al.*, 2013). The problem of the first classification of Saaremaa virus was also supported by our study results.

Phylogenetic analyses, mainly of S and M segment, of the newly obtained DOBV-positive specimen from Čermeľ- district of the town Košice (Eastern Slovakia) suggest that the newly obtained specimen of DOBV (SVK/22769/Aa) detected in striped field mouse (*A*. agrarius) is most closely related to the strains which have been previously detected in the same area (Klempa *et al.*, 2004; Klempa *et al.*, 2005). In the S segment as well as in the M segment tree, our new specimen (SVK/22769/Aa) obtained from the host *A. agrarius* is clustered within the "Kurkino" genotype which was defined according to the first sequences of the DOBV-Aa lineage found in the Kurkino region of Russia (Plyusnin *et al.*, 1999) and corresponds to the DOBV-Aa lineage on the European mainland (Klempa *et al.*, 2013).

According to our phylogenetic trees, for S segment as well as for M segment, all the sequences of DOBV obtained from *A. agrarius* from Slovakia are clustered within the clade representing the Kurkino serotype. On the other hand, all the Slovak sequences from the yellow necked mouse (*A. flavicollis*) are quite distanced in the clade

of Dobrava serotype. Thus, all the Slovak DOBV lineages were well separated according to their reservoir host species together with the virus serotypes. On the other hand, in the S segment tree, there have appeared at least two sequences from Slovenia from the *A. flavicollis* among the sequences from *A. agrarius* within the Kurkino genotype clade. Such spill-over infections have been observed also in Croatia (Plyusnina *et al.*, 2011). The opposite situation, the detection of DOBV-Aa strains in *A. flavicollis* could be recently observed in northern Germany (Schlegel *et al.*, 2009; Popugaeva *et al.*, 2012)

DOBV is considered as one of the most important origin of HFRS in humans in Europe (Sibold *et al.*, 2001; Klempa *et al.*, 2004). While DOBV-Aa seems to be dominant in Central Europe, DOBV-Af is mostly occurring serotype of DOBV in Balkan, even though the geographical range of *A. flavicollis* is almost over the whole European continent (Montgomery, 1999).

It is highly interesting to note that the different genotypes of DOBV (DOBV-Aa and DOBV-Af), even though they are highly genetically similar, they induce the HFRS disease of different severity. The most severe clinical courses have been reported in SE Europe, where the major part of human infections are caused by the Dobrava genotype of DOBV. The case fatality rate (CFR) 10-12 %, which is a rate similar to those infections caused by HTNV in Asia (Avsic-Zupanc *et al.*, 1999; Papa and Antoniadis, 2001; Vaheri *et al.*, 2013).

5.3 Bacteriophage MS2 as an internal control in duplex RT-PCR assay for the screening of hantaviruses

Reverse transcription (RT)-PCR assay as a diagnostic tool is widely used in detection of various RNA viruses from the families *Flaviviridae, Bunyaviridae* and *Orthomyxoviridae* (Romeiro *et al.*, 2016; Klempa *et al.*, 2006; Yanagita *et al.*, 2015). An advantage of RT-PCRs is a relatively fast detection of the pathogens' genome in clinical specimens such as blood, saliva or pieces of tissues since the onset of the first symptoms of illness (Saksida *et al.*, 2008). In general, a limitation of RT-PCRs for diagnostics is the fact that the test is positive only in the acute phase of disease. Especially hantavirus infections in humans often reaches low titer and can be missed

even with the most sensitive PCR methods (Krüger *et al.*, 2015). Another problem might be a possible presence of inhibitors of amplification which could be responsible for false-negative results. Therefore, for avoiding these mistakes was born an idea to use another template of nucleic acid in the PCR assay which serves as an internal control (IC). According to recommendations of some authors, IC should be noninfectious, clean from clinical specimens and genetically stable (Dreier *et al.*, 2005). Dipending on purposes, IC may be endogenous, as a naturally occuring genomic part within the analyzed specimen. On the other hand, exogenous IC is added before nucleic acid extraction and the coamplification is then performed within the same PCR reaction. The IC ia an important quality control of the procedure and has been described previously in early PCR experiments (Kim *et al.*, 2002; Hoorfar *et al.*, 2004).

In this study, we utilized a part of the protocol of duplex RT-PCR assay using bacteriophage MS2 as an exogenous IC originally developed to aim and detect hepatitis C virus RNA in blood donor screening (Dreier *et al.*, 2005). This assay was originally designed as the RT-PCR assay of the only one round of cycles, so we modified it by designing a novel second pair of primers annealing on the first PCR product of the phage MS2 RNA: "MS2:2781F" and "MS2: 2989R" for the second round of (semi-) nested PCR cycles (Table). There have been developed a couple of different PCR assays for hantavirus screening (Klempa *et al.*, 2006; Kramski *et al.*, 2007; Lagerqvist *et al.*, 2016; Kim *et al.*, 2016). We implemented the series of primers for MS2 phage into the nested Pan-Hanta-L-PCR assay, which was originally developed to detect and identify the all species of hantaviruses which are currently known and also those which are not discovered, yet. A set of degenerated primers (HANL-F1 and HANL-R1 for the first round of PCR and HANL-F2 and HANL-R2 for the nested PCR) were designed from an alignment of all available nucleotide sequences of the highly conserved part of L segment (Klempa *et al.*, 2006). We modified the Pan-Hanta-PCR assay by adding a set of primers for the bacteriophage MS2 RNA within the same RT-PCR reactions. The novel set of primers in this study was designed by utilizing Geneious software (Auckland, New Zealand).

5.4 Antiviral and antiproliferative activity of aryl-piperazine derivates

In general, chemical compounds of the class of aryl-piperazines, piperazines and their derivates, are considered as the most potent and broad-spectrum inhibitors of pathogens (Chaundhary *et al.*, 2006; Singh *et al.*, 2011; Kossakowski *et al.*, 2009; Bielenica *et al*, 2011; Ibezim *et al.*, 2012; Cianci *et al.*, 2013; Liu *et al.*, 2012). N-alkyl- and N-aryl-piperazine derivates were reported to have from low to moderate inhibitory activity against bacteria from the genera *Staphylococcus* and *Streptococcus*, and against *Pseudomonas aeruginosa* and *Escherichia coli*. A strong antifungal potential against a broad spectrum of fungi from the genus *Aspergillus* was reported within the same studies (Chaundhary *et al.*, 2006; Singh *et al.*, 2011). It has been reported that aryl-piperazine derivates are also potent inhibitors of pathogenic protozoal parasites, such as *Plasmodium falciparum*, one of the agents causing malaria (Ibezim *et al.*, 2012).

With respect to antiviral activities, the chemical group of aryl-piperazines showed a great antiviral potential against a broad spectrum of viruses, (–)ssRNA viruses including. It has been reported that a group of aryl-piperazine amides moderately inhibit the nucleoprotein (NP) of influenza A virus (Cianci *et al.*, 2013; Davis *et al.*, 2014). Interesting results were also brought to light by a study testing a novel piperazine derivate against HIV-1, which identified the CCR5-mediated fusion process as the molecular target of the compound (Liu *et al.*, 2012).

Therefore, considering that aryl-piperazine derivates appear to posses a great biological potential against pathogens in general, and against viruses in particular, we deemed interesting to investigate their inhibitory potential against two representatives of hantaviruses, also object of the molecular epidemiology study of this thesis. Hantaviruses PUUV and SEOV circulating in Europe represent two origins of HFRS (besides of DOBV) in humans. As far as we know, this study represents the first investigation of the antiviral activity of this class of chemical compounds against hantaviruses. Although none of the derivates showed any activity against these viruses, an interesting activity against Coxsackie virus B type 5 (CVB-5), the etiologic agent of often serious human infections, have been highlighted. In a previous work (Kossakowski *et al.*, 2009), other aryl-piperazine derivates were reported to inhibite type 2 of Coxsackie virus B (CVB-2). It is extremely interesting to underly that the aryl-

piperazine derivates found active against CVB-2 by the Kossakowski's group, were structurally similar to those we found active against CVB-5 in this study, specifically the phenyl-piperazine group and 4-chlorophenyl piperazine group. Three years later, another study tested a different set of aryl-piperazine derivates (Bielenica *et al.*, 2011). Also in this case, the activity of some derivates was confirmed against CVB-2. It is highly interesting that the active aryl-piperazine derivates contained the phenyl-piperazine group (**h**) and 4-fluorophenyl-piperazine group (**j**), the substitutions which seemed to be responsible for the biological activity also in this thesis. Thus, we could state that aryl-piperazine derivates generally have a great potential of antiviral effect against different serotypes of Coxsackie viruses of the B group.

CVB-5 serotype represents a serious pathogen known mainly as a causative agent of myocarditis, colds, cardiomyopathy, diabetes, neurological disorders, and inflammation. CVB-5 infection have caused widespread nationwide epidemics in the United States, particularly in 1961, 1972, and 1983. A great number of picornavirus inhibitors have been quite well described *in vitro*, but only few of them were active also *in vivo*; only Arbidol as an antivirotic compound developed in Russia, is available for the clinical use (Zhong *et al.*, 2009).

CONCLUSIONS

This study provides the first molecular data on PUUV from Czech Republic and considerably extends the available PUUV seuquence data from Slovakia and Germany. Identification of the virus clearly indicates a putative risk for human health in the Šumava and Eastern Slovakia even though these are currently not considered as hotspots for human hantavirus infections. The close phylogenetic relationships of the starins from Šumava, where no PUUV outbreaks are reported, with the strains obtained during the human PUUV outbreaks in the Bavarian Forrest (Schilling *et al.,* 2007; Essbauer *et al.*, 2006; Thoma *et al.*, 2014) confirm that more complex ecological and/or epidemiological factors than simply a presence of the virus itself are required for a development of outbreak situations (Drewes *et al.*, 2016). Our observations emphasize the benefits of obtaining sequence information of longer genomic stretches and form all genome segments. While longer sequences enable to more reliably and precisely estimate evolutionary relationships and facilitate the detection of clock signal (Biek *et al.*, 2015), allowing to time-calibrate the more recent period of the PUUV evolutionary history, having sequence information from all three segments opens new possibilities for studying the frequency of segment reassortments and its relevance for the PUUV emergence dynamics.

Our results of phylogenetic analyses highlight the necessity of sequencing all three hantavirus genome segments and of rodents screening not only in recognized endemic foci but also in regions with no reported human hantavirus disease cases. In DOBV phylogenetic analyses as well as in the case of PUUV, longer nucleotide sequences, particularly from M and L segment would allow to construct more balanced datasets for all genomic segments, which could be helpful to understand the complexity of DOBV lineages evolution better than now.

A set of seventeen aryl-piperazine derivates, novel in their structure and conformation, were tested for their biological activity against the replication of different representatives of the major RNA and DNA virus families, including two hantaviruses, as well as against a wide panel of human cancer derived cell lines.

Results show that some of these chemical compounds have selective activity against CVB-5 replication and proliferation of some tumoral cells. Analyses of the structure-activity relationships (SAR) indicate that aryl-piperazine derivates having a terminal phenyl-group associated with a linear hydrocarbon butyl-group is the most potent not only as the CVB-5 inhibitor, but as an inhibitor of different types of tumoral cells, too. It is important to note that the presence of halogens, such as fluorine and chlorine, substituting hydrogen within the cyclic hydrocarbon chain on the 4^{th} carbon on the terminal phenyl-group, in various combinations of the linear hydrocarbon chain binding the aryl-piperazine group, emerged as a promising antiviral or/and anticancer compound worthy of further investigation to determine its mode(s) of action, characteristic of resistant mutants, and effectiveness in animal models against CVB infections and tumoral growth.

Even though we identified parts of the molecules which are most likely responsible for their biological activity, it is not less important to identify the target of their activity on the observed organism. That would be the next step. The most commonly practiced approach in this future step is the selection of a resistant mutant. It starts by adding an active chemical compound in a very low concentration which allows to grow to non-resistant strains, too. The concentration of active compound is then gradually increased by the generations of the cells/virus until there is only a resistant mutant present which had enough time to grow in a sufficient viable colony. Next, a genome sequence information of the resistant mutant is obtained and compared with original non-resistant strain (or wild type) and the different DNA/RNA fragment is the target of acitivity.

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Internet resources:

web source 1: www.who.int/iris/bitstream/10665/.../9789241564540_eng.pdf

web source 2:

https://talk.ictvonline.org/files/ictv_official_taxonomy_updates_since_the_8th_report/m/animal-dsrna-and-ssrna--viruses/6640

web source3: https://talk.ictvonline.org/taxonomy/

web source 4: http://www.who.int/mediacentre/factsheets/fs164/en/

web source 5: https://www.cdc.gov/zika/geo/

web source 6: http://viralzone.expasy.org/all_by_species/24.html

web source 7: https://www.cdc.gov/yellowfever/transmission/index.html

web source 8: https://www.cdc.gov/westnile/resources/pdfs/BirdSpecies1999-2012.pdf

web source 9: https://www.cdc.gov/westnile/transmission/index.html

web source 10: http://www.who.int/tdr/publications/documents/dengue-diagnosis.pdf

web source 11: https://www.cdc.gov/yellowfever/vaccine/

web source 12:

https://wwwnc.cdc.gov/travel/yellowbook/2016/infectious-diseases-related-to-travel/yellow-fever

APPENDIX 1

Table 12: List of used aryl-piperazine derivates

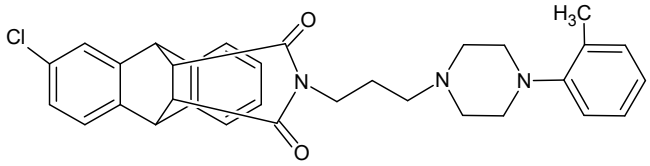
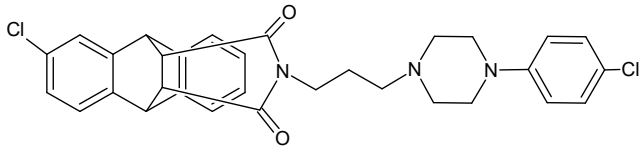
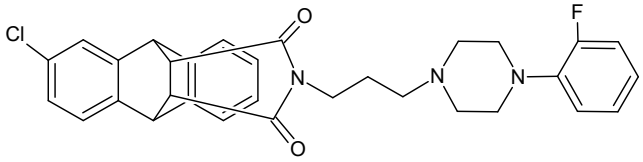
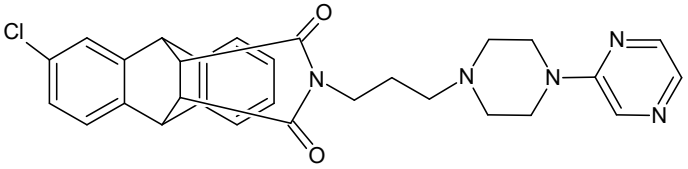
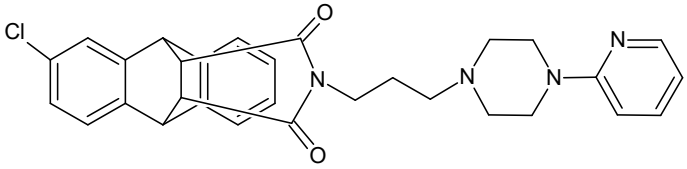
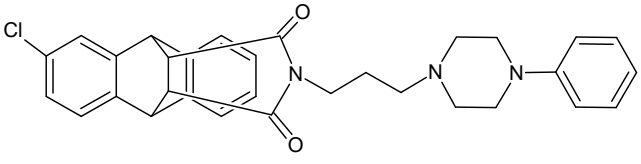
Symbol	Structure	Molar mass
2Cl3C1		526.06
2Cl3C2		546.48
2Cl3C3		530.03
2Cl3C5		514.02
2Cl3C6		513.03
2Cl3C8		512.04

Table 12: continuation List of used aryl-piperazine derivates

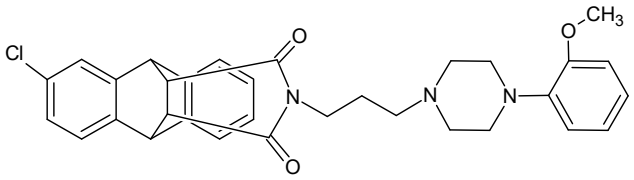
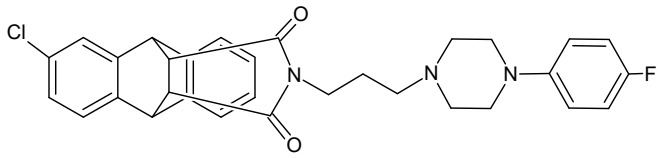
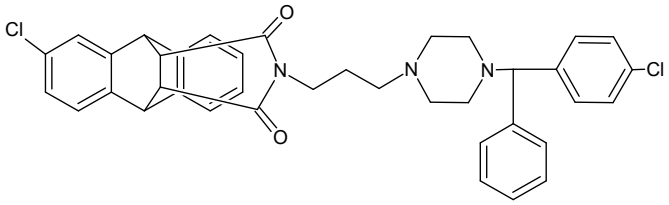
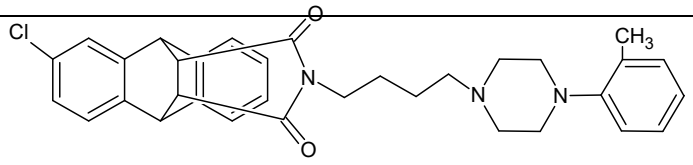
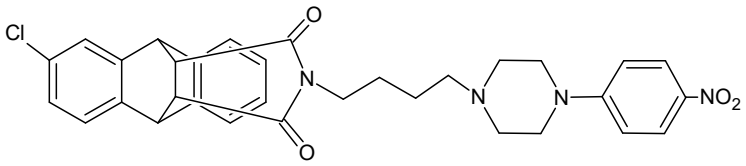
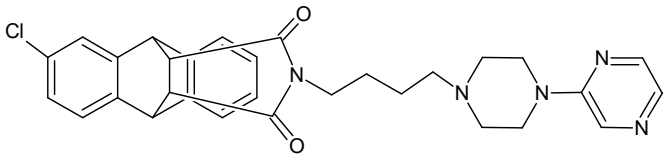
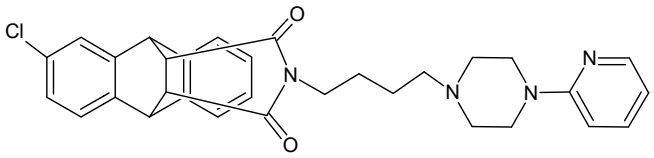
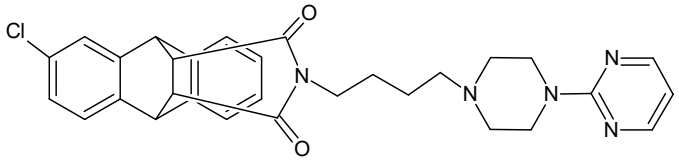
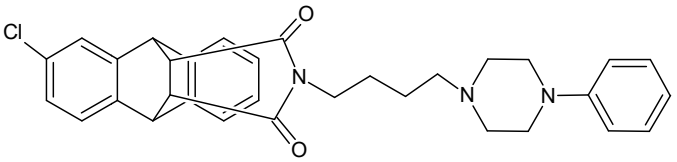
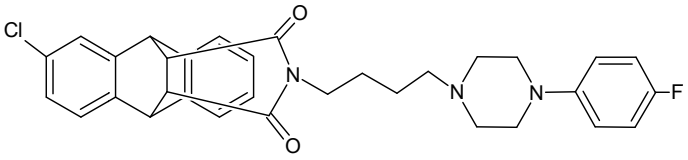
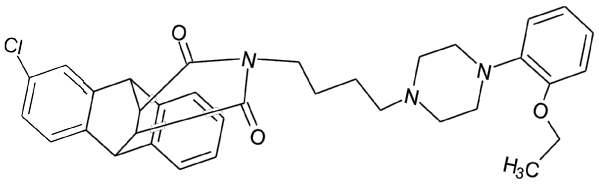
2Cl3C9		542.07
2Cl3C10		530.03
2Cl3C13		636.61
2Cl4C1		540.09
2Cl4C4		571.07
2Cl4C5		528.04

Table 12: continuation List of used aryl-piperazine derivates

2Cl4C6		527.06
2Cl4C7		528.04
2Cl4C8		526.07
2Cl4C10		544.06
2Cl4C12		570.12

APPENDIX 2

Table 13: List of title sequences used in phylogenetic and sequence analyses

Acc. No.	Strain (Isolate)	Reference
L segment tree DOBV/PUUV		
KU529945	Sochi/hu	Krautkramer *et al.* , 2016
KT315634	Igneada/89Af/2009	Polat *et al.*, 2015
KT315632	Igneada/57Af/2009	Polat *et al.*, 2015
KT315630	Igneada/13Af/2009	Polat *et al.*, 2015
KT315629	Igneada/09Af/2009	Polat *et al.*, 2015
KJ425422	Mu05_0239	Hofmann *et al.*, 2014
KJ182938	Mu08_0118	Hofmann *et al.*, 2014
KJ182937	Mu07_0293	Hofmann *et al.*, 2014
KF536035	Richtenberg/H827/12	Hofmann *et al.*, 2014
KF536033	Neuruppin/H431/10	Hofmann *et al.*, 2014
KF536032	Westergellersen/H91/08	Hofmann *et al.*, 2014
KF536031	Stockelsdorf/H90/08	Hofmann *et al.*, 2014
KF425496	LT-RS2	Nikolic *et al.*, 2013
KF425497	LT-RS3	Nikolic *et al.*, 2013
KF425495	LT-RS1	Nikolic *et al.*, 2013
JQ026206	GRW/Aa	Popugaeva *et al.*, 2012
GU904042	Slo/Af-BER	Kirsanovs *et al.*, 2010
GU904039	SK/Aa	Kirsanovs *et al.*, 2010
KM192209	Ap/Sochi/43	Kruger *et al.*, 2015
KM192208	Ap/Sochi/79	Kruger *et al.*, 2015
KM192207	Sochi/6882/hu	Kruger *et al.*, 2015
KT315633	Igneada/84Af/2009	Polat *et al.*, 2015
KT315631	Igneada/32Af/2009	Polat *et al.*, 2015
KT315628	Igneada/03Af/2009	Polat *et al.*, 2015
KT315627	Igneada/02Af/2009	Polat *et al.*, 2015
KP878310	10752/hu	Kruger *et al.*, 2015
KP878309	10645/Ap	Kruger *et al.*, 2015
KP878308	10636/Ap	Kruger *et al.*, 2015
KT885041	Stamforrad/POR	Miles & Lewandowski, 2015
KF776735	HtSi_375_p2012	Korva *et al.*, 2013
KF776720	HtSi_299_p2010	Korva *et al.*, 2013
KF776718	HtSi_286_p2009	Korva *et al.*, 2013
KF776717	HtSi_279_p2008	Korva *et al.*, 2013
KF776716	HtSi_275_p2008	Korva *et al.*, 2013
KF776715	HtSi_273_p2008	Korva *et al.*, 2013
KF776714	HtSi_270_p2008	Korva *et al.*, 2013
KF776709	HtSi_239_p2008	Korva *et al.*, 2013

Table 13: continuation

Acc. No.	Strain (Isolate)	Reference
L segment tree DOBV/PUUV		
KF776707	HtSi_231_p2007	Korva *et al.*, 2013
KF776706	HtSi_207_p2005	Korva *et al.*, 2013
KF776705	HtSi_130_p2000	Korva *et al.*, 2013
KF776704	HtSi_1033_a2001	Korva *et al.*, 2013
KF776703	HtSi_1034_a2001	Korva *et al.*, 2013
KF776702	HtSi_1013_a2002	Korva *et al.*, 2013
KF776701	HtSi_1014_a2002	Korva *et al.*, 2013
KF776700	HtSi_1042_a2008	Korva *et al.*, 2013
KF776698	HtSi_1009_a1995	Korva *et al.*, 2013
KF776697	HtSi_1043_a1995	Korva *et al.*, 2013
KF776696	HtSi_1040_a1999	Korva *et al.*, 2013
KF776695	HtSi_1016_a1995	Korva *et al.*, 2013
KF776694	HtSi_1017_a1995	Korva *et al.*, 2013
KF776693	HtSi_1015_a1995	Korva *et al.*, 2013
KF776692	HtSi_1021_a1995	Korva *et al.*, 2013
KF776690	HtSi_1039_a1996	Korva *et al.*, 2013
KF776689	HtSi_1026_a1995	Korva *et al.*, 2013
KF776688	HtSi_1028_a1995	Korva *et al.*, 2013
KF776687	HtSi_1029_a1995	Korva *et al.*, 2013
KF776686	HtSi_1027_a1995	Korva *et al.*, 2013
KF776685	HtSi_1032_a2001	Korva *et al.*, 2013
KF776653	HtSi_1030_a1995	Korva *et al.*, 2013
KF776649	HtSi_1037_a2001	Korva *et al.*, 2013
KF776648	HtSi_1035_a2002	Korva *et al.*, 2013
KF776647	HtSi_1038_a2002	Korva *et al.*, 2013
KF776646	HtSi_1036_a2001	Korva *et al.*, 2013
KF776645	HtSi_1031_a2001	Korva *et al.*, 2013
KF776644	HtSi_1090_a2000	Korva *et al.*, 2013
KF776643	HtSi_1022_a2000	Korva *et al.*, 2013
KF776642	HtSi_1025_a2000	Korva *et al.*, 2013
KF776641	HtSi_1024_a2000	Korva *et al.*, 2013
KF776640	HtSi_1023_a2000	Korva *et al.*, 2013
KF776639	HtSi_1019_a2007	Korva *et al.*, 2013
KF776638	HtSi_1018_a2007	Korva *et al.*, 2013
KF776637	HtSi_1010_a2007	Korva *et al.*, 2013
KF776636	HtSi_1011_a2007	Korva *et al.*, 2013
KF776635	HtSi_1041_a2005	Korva *et al.*, 2013
KF776634	HtSi_1012_a1997	Korva *et al.*, 2013

Table 13: continuation

Acc. No.	Strain (Isolate)	Reference
L segment tree DOBV/PUUV		
KF776632	HtSi_1097_a1995	Korva *et al.*, 2013
KF776631	HtSi_1091_a1997	Korva *et al.*, 2013
KF776633	HtSi_1092_a1997	Korva *et al.*, 2013
KF776630	HtSi_1096_a1995	Korva *et al.*, 2013
KF776629	HtSi_1095_a1997	Korva *et al.*, 2013
KF776628	HtSi_1094_a1998	Korva *et al.*, 2013
KF776627	HtSi_1008_a1999	Korva *et al.*, 2013
KF776626	HtSi_1044_a1999	Korva *et al.*, 2013
KF776624	HtSi_1020_a2008	Korva *et al.*, 2013
KF776623	HtSi_193_p2005	Korva *et al.*, 2013
KF776620	HtSi_301_p2010	Korva *et al.*, 2013
KF776619	HtSi_297_p2010	Korva *et al.*, 2013
KF776618	HtSi_268_p2008	Korva *et al.*, 2013
KF776615	HtSi_256_p2008	Korva *et al.*, 2013
KF776614	HtSi_215_p2005	Korva *et al.*, 2013
KF776613	HtSi_200_p2005	Korva *et al.*, 2013
KF776610	HtSi_135_p2001	Korva *et al.*, 2013
KF776609	HtSi_132_p2000	Korva *et al.*, 2013
KF776600	HtSi_300_p2010	Korva *et al.*, 2013
KF776597	HtSi_276_p2008	Korva *et al.*, 2013
KF776596	HtSi_274_p2008	Korva *et al.*, 2013
KF776590	HtSi_1093_a1996	Korva *et al.*, 2013
KF776586	HtSi_232_p2007	Korva *et al.*, 2013
KF776585	HtSi_221_p2006	Korva *et al.*, 2013
KF776583	HtSi_504_p2012	Korva *et al.*, 2013
KF776582	HtSi_498_p2012	Korva *et al.*, 2013
KF776581	HtSi_492_p2012	Korva *et al.*, 2013
KF776578	HtSi_465_p2012	Korva *et al.*, 2013
KF776564	HtSi_397_p2012	Korva *et al.*, 2013
KF776557	HtSi_353_p2012	Korva *et al.*, 2013
KF039740	Istanbul/H56/10	Ettinger *et al.*, 2013
KF177177	DOBV_RAV71	Stanojevic *et al.*, 2013
KF177176	DOBV_RAV69	Stanojevic *et al.*, 2013
JF920148	Ap/Sochi/hu	Dzagurova *et al.*, 2012
NC_005235	DOBV/Ano-Poroia/Afl9/1999	Nemirov *et al.*, 2002
AJ410617	DOBV/Ano-Poroia/Afl9/1999	Nemirov *et al.*, 2002
AB297667	DTK/Ufa-97	Abu Daude *et al.*, 2008

Table 13: continuation

Acc. No.	Strain (Isolate)	Reference
L segment tree DOBV/PUUV		
AB574183	Samara_49/CG/2005	Seto *et al*., 2011
AB574184	Samara_94/CG/2005	Seto *et al*., 2011
AY526217	Umea/hu	Johansson *et al*., 2004
EF405801	Kazan	Nemirov *et al.*, 2003
JN831945	PUUV/Pieksamaki/Mg7/2008	Plyusnina *et al*., 2012
KF776554	HtSi_369_p2012	Korva *et al*., 2013
KF776555	HtSi_368_p2012	Korva *et al*., 2013
KF776556	HtSi_335_p2012	Korva *et al*., 2013
KF776558	HtSi_357_p2012	Korva *et al*., 2013
KF776559	HtSi_437_p2012	Korva *et al*., 2013
KF776560	HtSi_341_p2012	Korva *et al*., 2013
KF776561	HtSi_374_p2012	Korva *et al*., 2013
KF776562	HtSi_377_p2012	Korva *et al*., 2013
KF776563	HtSi_379_p2012	Korva *et al*., 2013
KF776566	HtSi_400_p2012	Korva *et al*., 2013
KF776567	HtSi_402_p2012	Korva *et al*., 2013
KF776568	HtSi_410_p2012	Korva *et al*., 2013
KF776573	HtSi_417_p2012	Korva *et al*., 2013
KF776579	HtSi_483_p2012	Korva *et al*., 2013
KF776580	HtSi_485_p2012	Korva *et al*., 2013
KF776584	HtSi_315_p2011	Korva *et al*., 2013
KF776591	HtSI_1098_a2005	Korva *et al*., 2013
KF776593	HtSi_137_p2001	Korva *et al*., 2013
KF776594	HtSi_254_p2008	Korva *et al*., 2013
KF776595	HtSi_290_p2010	Korva *et al*., 2013
KF776599	HtSi_280_p2008	Korva *et al*., 2013
KF776602	HtSi_342_p2012	Korva *et al*., 2013
KF776603	HtSi_390_p2012	Korva *et al*., 2013
KF776605	HtSi_419_p2012	Korva *et al*., 2013
KF776616	HtSi_269_p2008	Korva *et al*., 2013
KF776617	HtSi_250_p2008	Korva *et al*., 2013
KF776622	HtSi_283_p2009	Korva *et al*., 2013
KF776650	HtSi_1061_a2002	Korva *et al*., 2013
KF776651	HtSi_1068_a1998	Korva *et al*., 2013
KF776652	HtSi_1063_a1999	Korva *et al*., 2013
KF776655	HtSi_1070_a1999	Korva *et al*., 2013
KF776656	HtSi_1076_a2006	Korva *et al*., 2013

Table 13: continuation

Acc. No.	Strain (Isolate)	Reference
L segment tree DOBV/PUUV		
KF776657	HtSi_1069_a1999	Korva *et al*., 2013
KF776658	HtSi_1072_a1999	Korva *et al*., 2013
KF776659	HtSi_1067_a1998	Korva *et al*., 2013
KF776660	HtSi_1071_a1999	Korva *et al*., 2013
KF776662	HtSi_1065_a1996	Korva *et al*., 2013
KF776663	HtSi_1066_a1996	Korva *et al*., 2013
KF776668	HtSi_1050_a1995	Korva *et al*., 2013
KF776670	HtSi_1057_a1995	Korva *et al*., 2013
KF776677	HtSi_1045_a1993	Korva *et al*., 2013
KF776678	HtSi_1046_a1993	Korva *et al*., 2013
KF776679	HtSi_1060_a1993	Korva *et al*., 2013
KF776680	HtSi_1048_a1993	Korva *et al*., 2013
KF776691	HtSi_1077_a2011	Korva *et al*., 2013
KF776708	HtSi_235_p2007	Korva *et al*., 2013
KF776710	HtSi_246_p2008	Korva *et al*., 2013
KF776711	HtSi_247_p2008	Korva *et al*., 2013
KF776713	HtSi_267_p2008	Korva *et al*., 2013
KF776719	HtSi_293_p2010	Korva *et al*., 2013
KF776721	HtSi_336_p2012	Korva *et al*., 2013
KF776723	HtSi_346_p2012	Korva *et al*., 2013
KF776724	HtSi_355_p2012	Korva *et al*., 2013
KF776725	HtSi_363_p2012	Korva *et al*., 2013
KF776726	HtSi_366_p2012	Korva *et al*., 2013
KF776727	HtSi_384_p2012	Korva *et al*., 2013
KF776728	HtSi_387_p2012	Korva *et al*., 2013
KF776730	HtSi_403_p2012	Korva *et al*., 2013
KF776731	HtSi_418_p2012	Korva *et al*., 2013
KJ994778	Mu/07/1219	Ali *et al*., 2014
M63194	CG1820	Stohwasser *et al*., 1991
NC_005225	Sotkamo	Piiparinen *et al*., 2011
JX028271	1.nov	Lee *et al.*, 2014
JX046485	5.nov	Lee *et al.*, 2014
JX046482	4.nov	Lee *et al.*, 2014
JX131293	21.nov	Lee *et al.*, 2014
JX131290	19.nov	Lee *et al.*, 2014
JX131287	16.nov	Lee *et al.*, 2014
EF646763	PH-1	Song *et al.*, 2007

Table 13: continuation

Acc. No.	Strain (Isolate)	Reference
L segment tree DOBV/PUUV		
JX316008	B41	Pounder,K.C. et al., 2013
JF915740	YN05-7	Zuo,S.-Q. et al., 2011
AB677488	Khekhtsir37L/2002	Kariwa,H. et al., 2012
NC_003468	Chile-9717869	Meissner J. D. et al., 2002
JX443696	H731172/BRA259	Firth C. et al., 2012
NC_005217	NM H10	Chizhikov,V.E. et al., 1995
KP013558	Mm/173-11	Tkachenko *et al.*, 2015
KP013565	Mm/5779-09	Tkachenko *et al.*, 2015
KP013561	Mm/340-08	Tkachenko *et al.*, 2015
KP013560	Mm/302-08	Tkachenko *et al.*, 2015
KP013563	Mm/560-11	Tkachenko *et al.*, 2015
KP013559	Mm/296-08	Tkachenko *et al.*, 2015
KP013562	Mm/554-11	Tkachenko *et al.*, 2015
KP013556	Mm/98-08	Tkachenko *et al.*, 2015
KP013564	Mm/603-11	Tkachenko *et al.*, 2015
KP013557	Mm/172-11	Tkachenko *et al.*, 2015
KP013566	Mm/5844-09	Tkachenko *et al.*, 2015
KP013567	Ma/5990-09	Tkachenko *et al.*, 2015
HQ728466	GER/125/Marv	Schlegel *et al.*, 2012
NC_005226	Moravia/5302v/95	Kukkonen *et al.*, 1998
HQ728463	GER/39/Marv	Schlegel *et al.*, 2012
HQ728461	GER/08/525/Marv	Schlegel *et al.*, 2012
HQ728455	CH/09/1026/Arv	Schlegel *et al.*, 2012
HQ728457	GER/109/Arv	Schlegel *et al.*, 2012
HQ728459	GER/152/Arv	Schlegel *et al.*, 2012
HQ728462	GER/20/Marv	Schlegel *et al.*, 2012
HQ728453	GER/08/712/Arv	Schlegel *et al.*, 2012
HQ728454	GER/09/815/Arv	Schlegel *et al.*, 2012
FJ495100	TULV/Sestrze/Mag98_02	Korva *et al.*, 2009
FJ495102	TULV/Sred ob Dravi/Ms51_97	Korva *et al.*, 2009
NC_005222	76-118	Schmaljohn, 1990
JQ082302	SA14	Klempa *et al.*, 2012
NC_005238	80-39	Antic *et al.*, 1991

Table 13: continuation

Acc. No.	Strain (Isolate)	Reference
S segment tree PUUV		
AB010730	Kamiiso-8Cr-95	Kariwa *et al*, 1999
AB010731	Tobetsu-60Cr-93	Kariwa *et al*, 1999
AB011630	Vladivostok 1Mf95	Kariwa *et al*, 2008
AB297665	DTK/Ufa-97	Abu Daude *et al*, 2008
AB433842	Samara_6/CG/2005	Kariwa *et al*, 2009
AB433844	Samara_68/CG/2005	Kariwa *et al*, 2009
AB433845	Samara_94/CG/2005	Kariwa *et al*., 2009
AB675450	Tobetsu35S/2010	Kariwa *et al.*, 2012
AB675459	Kitahiyama77S/2008	Kariwa *et al.*, 2012
AB675462	Kitahiyama124S/2008	Kariwa *et al.*, 2012
AB675463	Kitahiyama128S/2008	Sanada *et al*., 2012
AB675464	Tobetsu89S/2000	Kariwa *et al.*, 2012
AB675466	Tobetsu57S/2004	Kariwa *et al.*, 2012
AB675469	Ishikari9S/2009	Kariwa *et al.*, 2012
AB675471	Nakagawa49S/2004	Kariwa *et al.*, 2012
AB675472	Nakagawa73S/2004	Kariwa *et al.*, 2012
AB675474	Kiritappu126S/2000	Kariwa *et al.*, 2012
AB675477	Shari6-10S/2010	Kariwa *et al.*, 2012
AB675478	ShariP5-3S/2010	Kariwa *et al.*, 2012
AB675479	ShariG2-1S-As/2010	Kariwa *et al.*, 2012
AB675480	Sakhaline96S/1998	Kariwa *et al.*, 2012
AF294652	Opina916	Leitmeyer *et al*., 2001
AF367064	CG144	Dekonenko *et al.*, 2003
AF367065	CG168	Dekonenko *et al.*, 2003
AF367066	CG215	Dekonenko *et al.*, 2003
AF367068	CG315	Dekonenko *et al.*, 2003
AF367069	CRF161	Dekonenko *et al.*, 2003
AF367070	CRF308	Dekonenko *et al.*, 2003
AF411446	F-s808	Dekonenko and Morozov, 2001
AF411447	CG142	Dekonenko *et al*., 2003
AF442613	CG17/Baskiria-2001	Dekonenko *et al*., 2003
AJ011646	Ls136V	Vapalahti *et al*., 1999
AJ223368	Puu/Eidsvoll/1124v	Lundkvist *et al*., 1998
AJ223369	Puu/Eidsvoll/Cg1138/87	Lundkvist *et al*., 1998
AJ223371	Puu/Huggberget/Cg36/94	Lundkvist *et al*., 1998
AJ223374	Puu/Mellansel/Cg47/94	Lundkvist *et al*., 1998
AJ223375	Puu/Mellansel/Cg49/94	Lundkvist *et al*., 1998

Table 13: continuation

Acc. No.	Strain (Isolate)	Reference
S segment tree PUUV		
AJ223376	Puu/Solleftea/Cg3/95	Lundkvist *et al*., 1998
AJ223377	Puu/Solleftea/Cg6/95	Lundkvist *et al*., 1998
AJ223380	Puu/Tavelsjo/Cg81/94	Lundkvist *et al*., 1998
AJ238779	PUU/Cg-Erft	Heiske *et al*., 1999
AJ238788	Karhumaki	Asikainen *et al*., 2000
AJ238789	Kolodozero	Asikainen *et al*., 2000
AJ238790	Gomselga	Asikainen *et al*., 2000
AJ238791	Fyn	Asikainen *et al*., 2000
AJ277030	Thuin/33Cg/96	Escutenaire *et al*., 2001
AJ277031	Montbliart/23Cg/96	Escutenaire *et al*., 2001
AJ277032	Momignies/47Cg/96	Escutenaire *et al*., 2001
AJ277033	Momignies/55Cg/96	Escutenaire *et al*., 2001
AJ277034	Couvin/59Cg/97	Escutenaire *et al*., 2001
AJ277075	CG14444	Escutenaire *et al*., 2001
AJ277076	CG14445	Escutenaire *et al*., 2001
AJ278092	Fyn131	Asikainen *et al*., 2000
AJ278093	Fyn131	Asikainen *et al*., 2000
AJ314597	Pallasjarvi/63Cg/98	Sironen *et al*., 2001
AJ314598	Baltic/49Cg/00	Sironen *et al*., 2001
AJ314599	Baltic/205Cg/00	Sironen *et al*., 2001
AJ314600	Balkan-1	Sironen *et al*., 2001
AJ314601	Balkan-2	Sironen *et al*., 2001
AJ888751	PUU/Klippitztoerl/Cg9/1995	Plyusnina *et al*., 2006
AJ888752	PUU/Ernstbrunn/Cg641/1995	Plyusnina *et al*., 2006
AM695638	PUU/Mignovillard/CgY02/2005	Plyusnina *et al*., 2007
AM746297	Bussjo_95-1	Johansson *et al*., 2008
AM746298	Bussjo_95-2	Johansson *et al*., 2008
AM746310	Djaknebole_98-1	Johansson *et al*., 2008
AM746311	Djaknebole_98-2	Johansson *et al*., 2008
AM746315	Gumboda_98-1	Johansson *et al*., 2008
AM746316	Gumboda_98-2	Johansson *et al*., 2008
AM746317	Gumboda_98-3	Johansson *et al*., 2008
AM746318	Gumboda_98-4	Johansson *et al*., 2008
AM746319	Gumboda_98-5	Johansson *et al*., 2008
AM746320	Norum_98-1	Johansson *et al*., 2008
AM746321	Norum_98-2	Johansson *et al*., 2008
AM746324	Norum_98-5	Johansson *et al*., 2008

Table 13: continuation

Acc. No.	Strain (Isolate)	Reference
S segment tree PUUV		
AM746325	Norum_98-6	Johansson *et al*., 2008
AM746328	Palbole_98-2	Johansson *et al*., 2008
AM746329	Palbole_98-3	Johansson *et al*., 2008
AM746331	Skaran_98-1	Johansson *et al*., 2008
AM930973	VLA/Barguzin/Mo483/2005	Plyusnina *et al*., 2008
AY197740	Fusong48	Liu *et al*., 2003
AY217102	Fushun28	Liu *et al*., 2003
AY954722	Bavaria CG 9/04	Essbauer *et al*., 2006
AY954723	Bavaria CG 34/04	Essbauer *et al*., 2006
AY954724	Bavaria CB 20/04	Essbauer *et al*., 2006
AY954725	Bavaria CG 2/04	Essbauer *et al*., 2006
DQ016430	Bavaria CG 33/04	Essbauer *et al*., 2006
DQ016431	Bavaria CG 39/04	Essbauer *et al*., 2006
DQ094844	Heidelberg/hu	Bahr *et al*., 2006
DQ138128	00-18	Song *et al*., 2007
DQ138133	96-1	Song *et al*., 2007
DQ138140	99-27	Song *et al*., 2007
DQ138142	99-28	Song *et al*., 2007
DQ322669	Cologne Mu05/241	Essbauer *et al*., 2007
DQ322672	Cologne Mu05/244	Essbauer *et al*., 2007
DQ322675	Cologne Mu05/258	Essbauer *et al*., 2007
DQ322678	Cologne Mu05/269	Essbauer *et al*., 2007
DQ322680	Cologne Mu05/272	Essbauer *et al*., 2007
DQ408268	Cologne Mu05/274	Essbauer *et al*., 2007
DQ408270	Cologne Mu05/277	Essbauer *et al*., 2007
DQ408271	Cologne Mu05/281	Essbauer *et al*., 2007
DQ408274	Cologne Mu05/286	Essbauer *et al*., 2007
DQ408275	Cologne Mu05/161	Essbauer *et al*., 2007
EF211819	Fusong 84-05	Tang and Li, 2007
EF442087	Fusong-Cr-247	Zhang *et al*., 2007
EF442091	Fusong-Cr-275	Zhang *et al*., 2007
EF488804	Fusong 114-05	Wu *et al*., 2007
EF488805	Fusong 843-06	Wu *et al*., 2007
EF488806	Fusong 900-06	Wu *et al*., 2007
EU004029	Bavaria/H139/04	Schilling *et al*., 2007
EU004030	Bavaria/H151/04	Schilling *et al*., 2007
EU004031	Bavaria/H201/04	Schilling *et al*., 2007

Table 13: continuation

Acc. No.	Strain (Isolate)	Reference
S segment tree PUUV		
EU004032	Bavaria/ClgM2/04	Schilling *et al*., 2007
EU004033	Bavaria/ClgM26/04	Schilling *et al*., 2007
EU004034	Bavaria/ClgM37/04	Schilling *et al*., 2007
EU004035	D208/04	Schilling *et al*., 2007
EU004036	Kobl3/05	Schilling *et al*., 2007
EU072480	Fusong-Mf-682	Zou *et al*., 2008
EU072481	Fusong-Mf-731	Zou *et al*., 2008
EU072482	Yakeshi-Mm-31	Zou *et al*., 2008
EU072483	Yakeshi-Mm-59	Zou *et al*., 2008
EU072484	Yakeshi-Mm-182	Zou *et al*., 2008
EU085558	Hemmingen13	Schmidt-Chanasit, 2007
EU085559	Weissach20	Schmidt-Chanasit, 2007
EU085563	Leonberg42	Schmidt-Chanasit, 2007
EU085565	Renningen50	Schmidt-Chanasit, 2007
EU085566	Renningen54	Schmidt-Chanasit, 2007
EU246962	Laufach133	Schmidt-Chanasit, 2007
EU246968	Rechtenbach85	Schmidt-Chanasit, 2007
EU266757	SJ/H101Westerstetten/07	Hofmann *et al*., 2008
EU266758	SJ/H233Stuttgart/07	Hofmann *et al*., 2008
EU266759	SJ/H99Steinheim/07	Hofmann *et al*., 2008
EU266760	SJ/H145Pfullingen/07	Hofmann *et al*., 2008
EU266761	SJ/H85Albstadt/07	Hofmann *et al*., 2008
EU266762	SJ/M4Albstadt/07	Hofmann *et al*., 2008
EU266763	SJ/H81Mossingen/07	Hofmann *et al*., 2008
EU266764	SJ/H232Sigmaringen/07	Hofmann *et al*., 2008
EU266765	SJ/H231Remseck/07	Hofmann *et al*., 2008
EU266766	BF/H72Waldkirchen/07	Hofmann *et al*., 2008
EU266767	SF/H290Schluchtern/07	Hofmann *et al*., 2008
EU266768	H127Karlsruhe/07	Hofmann *et al*., 2008
EU266769	ML/M837Billerbeck/07	Hofmann *et al*., 2008
EU266771	ML/H303Vreden/07	Hofmann *et al*., 2008
EU266772	H68Essen/07	Hofmann *et al*., 2008
EU439968	Bavaria 151/05	Essbauer *et al*., 2010
EU439969	Bavaria 152/05	Essbauer *et al*., 2010
EU439972	Bavaria 159/05	Essbauer *et al*., 2010
FJ170793	Yuanjiang-Mf-21	Zou *et al*., 2008
FJ170797	Shenyang-Mf-135	Zou *et al*., 2008

Table 13: continuation

Acc. No.	Strain (Isolate)	Reference
S segment tree PUUV		
FN377821	PUUV/Mg9/HungaryTR17/00	Plyusnina *et al*., 2009
FN377822	PUUV/Mg23/HungaryTR17/00	Plyusnina *et al*., 2009
GQ339473	Kiviniemi/Mg3/05	Nemirov *et al*., 2010
GQ339474	Kiviniemi/Mg5/05	Nemirov *et al*., 2010
GQ339477	Aijajarvi/Mg9/05	Nemirov *et al*., 2010
GQ339478	Jockfall/Mg12/05	Nemirov *et al*., 2010
GQ339479	Moskosel/Mg17/05	Nemirov *et al*., 2010
GQ339480	Gyttjea/Mg19/05	Nemirov *et al*., 2010
GQ339481	Ljustrask/Mg20/05	Nemirov *et al*., 2010
GQ339482	Kalvudden/Mg22/05	Nemirov *et al*., 2010
GQ339483	Bergsjobo/Mg25/05	Nemirov *et al*., 2010
GQ339484	Faboviken/Mg26/05	Nemirov *et al*., 2010
GQ339485	Mangelbo/Mg1/05	Nemirov *et al*., 2010
GQ339486	Munga/Mg2/05	Nemirov *et al*., 2010
GU300138	Sen05/107	Mertens *et al*., 2011
GU300143	Sen05/176	Essbauer *et al*., 2010
GU808824	Kuhmo/X5	Sironen *et al*., 2010
GU808825	Kuhmo/X11	Sironen *et al*., 2010
HE801633	Sotkamo 2009	Kurolt *et al.*, 2012
JN657228	PUUV/Jelgava/Mg149/2008	Razzauti *et al*., 2012
JN657229	PUUV/Madona/Mg/99/2008	Razzauti *et al*., 2012
JN657230	PUUV/Jelgava/Mg136/2008	Razzauti *et al*., 2012
JN657231	PUUV/Jelgava/Mg140/2008	Razzauti *et al*., 2012
JN696317	H400Samern/10	Ettinger *et al*., 2012
JN696318	H581Zenting/10	Ettinger *et al*., 2012
JN696319	H293Sinntal/10	Ettinger *et al*., 2012
JN696320	H186Sasbach/10	Ettinger *et al*., 2012
JN696321	H126Muenster/10	Ettinger *et al*., 2012
JN696322	H137Bissendorf/10	Ettinger *et al*., 2012
JN696323	H156Ostercappeln/10	Ettinger *et al*., 2012
JN696324	H163Friolzheim/10	Ettinger *et al*., 2012
JN696325	H179Pforzheim/10	Ettinger *et al*., 2012
JN696326	H203Aschaffenburg/10	Ettinger *et al*., 2012
JN696327	H244Wiesenbach/10	Ettinger *et al*., 2012
JN696328	H266Stuttgart/10	Ettinger *et al*., 2012
JN696329	H287Waldkirchen/10	Ettinger *et al*., 2012
JN696330	H288Niederstetten/10	Ettinger *et al*., 2012

Table 13: continuation

Acc. No.	Strain (Isolate)	Reference
S segment tree PUUV		
JN696331	H290Fulda/10	Ettinger *et al*., 2012
JN696333	H347Melsungen/10	Ettinger *et al*., 2012
JN696334	H357Bissendorf/10	Ettinger *et al*., 2012
JN696335	H358Ostercappeln/10	Ettinger *et al*., 2012
JN696336	H368Bad_Iburg/10	Ettinger *et al*., 2012
JN696337	H362Melle/10	Ettinger *et al*., 2012
JN696339	H372Bissendorf/10	Ettinger *et al*., 2012
JN696340	H373Osnabrueck/10	Ettinger *et al*., 2012
JN696341	H461Reutlingen/10	Ettinger *et al*., 2012
JN696342	H467Herrenberg/10	Ettinger *et al*., 2012
JN696344	H542Hoesbach/10	Ettinger *et al*., 2012
JN696345	H519Bad_Urach/10	Ettinger *et al*., 2012
JN696346	H510St.Johann/10	Ettinger *et al*., 2012
JN696348	H521Reken/10	Ettinger *et al*., 2012
JN696349	H524Eningen_unter_Achalm/10	Ettinger *et al*., 2012
JN696351	H538Deggendorf/10	Ettinger *et al*., 2012
JN696352	H564Tuebingen/10	Ettinger *et al*., 2012
JN696353	H569Eningen_unter_Achalm/10	Ettinger *et al*., 2012
JN696354	H574Stuttgart/10	Ettinger *et al*., 2012
JN696355	Mu727Astrup/07	Ettinger *et al*., 2012
JN696356	Mu522Sinntal/10	Ettinger *et al*., 2012
JN696357	Mu442Sinntal/10	Ettinger *et al*., 2012
JN696358	Mu362Osnabrueck/05	Ettinger *et al*., 2012
JN696360	Mu1054Heimerdingen2/10	Ettinger *et al*., 2012
JN696364	Mu2232Bramsche/09	Ettinger *et al*., 2012
JN696365	Mu3072Bramsche/10	Ettinger *et al*., 2012
JN696367	Mu428Darmstadt/09	Ettinger *et al*., 2012
JN696368	Mu557Gilserberg/10	Ettinger *et al*., 2012
JN696369	Mu563Salmuenster/10	Ettinger *et al*., 2012
JN696371	MuEb6Karlstadt/10	Ettinger *et al*., 2012
JN696372	MuEb4Karlstadt/10	Ettinger *et al*., 2012
JN696373	MuEb10Karlstadt/10	Ettinger *et al*., 2012
JN696374	MuEb12Lackenberg/10	Ettinger *et al*., 2012
JN696375	MuEb14Lackenberg/10	Ettinger *et al*., 2012
JN696376	MuEb51Elsenthal/10	Ettinger *et al*., 2012
JN831943	PUUV/Pieksamaki/Mg7/2008	Plyusnina *et al*., 2012
JQ319161	PUUV/Konnevesi/Mg_O57A/2005	Razzauti *et al*., 2012

Table 13: continuation

Acc. No.	Strain (Isolate)	Reference
S segment tree PUUV		
JQ319162	PUUV/Konnevesi/Mg_O78A/2005	Razzauti *et al*., 2012
JQ319163	PUUV/Konnevesi/Mg_M94A/2005	Razzauti *et al*., 2012
JQ319164	PUUV/Konnevesi/Mg_O6B/2005	Razzauti *et al*., 2012
JQ319166	PUUV/Konnevesi/Mg_O14B/2005	Razzauti *et al*., 2012
JQ319167	PUUV/Konnevesi/Mg_O15B/2005	Razzauti *et al*., 2012
JX028273	11_1	Lee *et al*., 2014
JX046484	11_4	Lee *et al*., 2014
JX046487	11_5	Lee *et al*., 2014
KC676609	PUUV/Croatia_Gerovo/Mg938/2008	Svoboda *et al*., 2014
KC676610	PUUV/Croatia_Gerovo/Mg954/2008	Svoboda *et al*., 2014
KF776854	HtSi_390_p2012	Korva *et al*., 2013
KF776855	HtSi_335_p2012	Korva *et al*., 2013
KF776856	HtSi_374_p2012	Korva *et al*., 2013
KF776858	HtSi_399_p2012	Korva *et al*., 2013
KF776859	HtSi_402_p2012	Korva *et al*., 2013
KF776860	HtSi_410_p2012	Korva *et al*., 2013
KF776861	HtSi_412_p2012	Korva *et al*., 2013
KF776863	HtSi_419_p2012	Korva *et al*., 2013
KF776864	HtSi_1054_a1995	Korva *et al*., 2013
KF776865	HtSi_424_p2012	Korva *et al*., 2013
KF776866	HtSi_434_p2012	Korva *et al*., 2013
KF776867	HtSi_440_p2012	Korva *et al*., 2013
KF776868	HtSi_483_p2012	Korva *et al*., 2013
KF776869	HtSi_485_p2012	Korva *et al*., 2013
KF776870	HtSi_493_p2012	Korva *et al*., 2013
KF776871	HtSi_317_p2012	Korva *et al*., 2013
KF776872	HtSi_1101_a2007	Korva *et al*., 2013
KF776877	HtSi_426_p2012	Korva *et al*., 2013
KF776878	HtSi_430_p2012	Korva *et al*., 2013
KF776879	HtSi_1063_a1999	Korva *et al*., 2013
KF776880	HtSi_1068_a1998	Korva *et al*., 2013
KF776881	HtSi_1058_a1995	Korva *et al*., 2013
KF776883	HtSi_1078_a1995	Korva *et al*., 2013
KF776885	HtSi_1071_a1999	Korva *et al*., 2013
KF776890	HtSi_142_p2002	Korva *et al*., 2013
KF776891	HtSi_199_p2005	Korva *et al*., 2013
KF776893	HtSi_246_p2008	Korva *et al*., 2013

Table 13: continuation

Acc. No.	Strain (Isolate)	Reference
S segment tree PUUV		
KF776894	HtSi_247_p2008	Korva *et al*., 2013
KF776895	HtSi_248_p2008	Korva *et al*., 2013
KF776898	HtSi_254_p2008	Korva *et al*., 2013
KF776899	HtSi_262_p2008	Korva *et al*., 2013
KF776900	HtSi_269_p2008	Korva *et al*., 2013
KF776901	HtSi_283_p2009	Korva *et al*., 2013
KF776903	HtSi_339_p2012	Korva *et al*., 2013
KF906512	KS13/855	Ali *et al*., 2014
KJ857342	Fuyuan-Mm-217	Wang *et al.*, 2014
KJ857343	Fuyuan-Mm-228	Wang *et al.*, 2014
KJ857344	Fuyuan-Mm-250	Wang *et al.*, 2014
KJ857345	Fuyuan-Mm-254	Wang *et al.*, 2014
KJ857346	Fuyuan-Mm-312	Wang *et al.*, 2014
L08804	K27	Xiao *et al*., 1993
L11347	P360	Xiao *et al*., 1993
L36943	Berkel	Pilaski *et al*., 1994
M32750	CG1820	Stohwasser *et al*., 1990
NC_005224	Sotkamo	Vapalahti *et al*., 1992
U14137	Vranica	Reip *et al*., 1995
U22423	CG 13891	Browen *et al*., 1995
U35255	MF-43	Horling *et al.*, 1996
U95306	Kl6	Bowen *et al*., 1997
U95307	P1	Bowen *et al*., 1997
U95308	Kl1	Bowen *et al*., 1997
Z21497	Udmurtia/894Cg/91	Plyusnin *et al*., 1995
Z30702	Evo/12Cg/93	Plyusnin *et al*., 1995
Z30704	Evo/14Cg/93	Plyusnin *et al*., 1995
Z30705	Evo/15Cg/93	Plyusnin *et al*., 1995
Z30706	Udmurtia/444Cg/88	Plyusnin *et al*., 1995
Z30707	Udmurtia/458Cg/88	Plyusnin *et al*., 1995
Z30708	Udmurtia/338Cg/92	Plyusnin *et al*., 1995
Z46942	Puu/Puu/1324Cg/79	Plyusnin *et al*., 1995
Z48586	Puu/Vindeln/L20Cg/83	Horling *et al*., 1995
Z69985	Puu/Virrat/25Cg/95	Plyusnin *et al*., 1997
Z84204	Puu/Kazan	Lundkvist *et al*., 1997
AM746330	Palbole_98-4	Johansson *et al.*, 2008
AM930972	HOK/Muhorshibir/Mr767/2005	Plyusnina *et al*., 2008

Table 13: continuation

Acc. No.	Strain (Isolate)	Reference
S segment tree PUUV		
DQ016432	Bavaria CG 41/04	Essbauer *et al.*, 2006
EU439971	Bavaria 157/05	Essbauer *et al.*, 2010
AY526219	Umea/hu	Johansonn *et al*., 2004
M segment tree PUUV		
AB433850	Samara_49/CG/2005	Kariwa *et al.,* 2009
AB433852	Samara_94/CG/2005	Kariwa *et al*., 2009
U14136	Vranica	Reip *et al*., 1995
AY526218	Umea/hu	Johansson *et al*., 2004
NC_005223	Sotkamo	Vapalahti *et al*., 1992
U22418	CG 13891	Bowen *et al*., 1995
EF198413	04-4	Song 2007
AB297666	DTK/Ufa-9	Abu Daude *et al.,* 2010
AF442617	CRF308/Omsk	Dekonenko *et al.,* 2003
AF442615	CG215/Omsk	Dekonenko *et al.,* 2003
AF442614	CG17/Baskiria-2001	Dekonenko *et al.,* 2003
AF367061	CRF161	Dekonenko *et al.,* 2003
JN831944	PUUV/Pieksamaki/Mg7/2008	Plyusnina *et al.*, 2012
JQ319175	PUUV/Konnevesi/Mg_M114B/2005	Razzauti *et al*., 2013
JQ319174	PUUV/Konnevesi/Mg_M78B/2005	Razzauti *et al*., 2013
JQ319173	PUUV/Konnevesi/Mg_M105A/2005	Razzauti *et al*., 2013
JQ319172	PUUV/Konnevesi/Mg_O78A/2005	Vapalahti *et al*., 1992
JX028272	11_1	Lee *et al.*, 2014
JX046486	11_5	Lee *et al.*, 2014
JX046483	11_4	Lee *et al.*, 2014
EU069361	11604	Mukhametkhanov and Baymiev, 2007
Z84205	Puu/Kazan	Lundkvist *et al.*, 2003
Z70201	Puu/Virrat/25Cg/95	Plyusnin *et al.*, 1997
Z49214	Puu/Vindeln/L20Cg/83	Horling *et al.*, 1995
AJ238778	PUU/Cg-Erft	Heiske *et al.*, 1999
KC676632	PUUV/Croatia_Gerovo/Mg978/2008	Svoboda *et al.*, 2013
KC676630	PUUV/Croatia_Gerovo/Mg954/2008	Svoboda *et al.*, 2013
KC676629	PUUV/Croatia_Gerovo/Mg938/2008	Svoboda *et al.*, 2013
DQ518237	Bavaria CG 41/04	Essbauer *et al.*, 2007
DQ518236	Bavaria CG 39/04	Essbauer *et al.*, 2007
DQ518233	Bavaria CG 152/05	Essbauer *et al.*, 2007
DQ518230	Cologne Mu05/269	Essbauer *et al.*, 2007
DQ518223	Bavaria CG 33/04	Essbauer *et al.*, 2007

Table 13: continuation

Acc. No.	Strain (Isolate)	Reference
M segment tree PUUV		
DQ518221	Cologne Mu05/258	Essbauer *et al.*, 2007
DQ518219	Bavaria CG 9/04	Essbauer *et al.*, 2007
DQ518218	Bavaria CG 2/04	Essbauer *et al.*, 2007
DQ518217	Osnabrueck Mu05/392	Essbauer *et al.*, 2007
DQ518216	Cologne Mu05/285	Essbauer *et al.*, 2007
DQ518215	Cologne Mu05/161	Essbauer *et al.*, 2007
DQ518213	Cologne Mu05/244	Essbauer *et al.*, 2007
DQ518211	Cologne Mu05/241	Essbauer *et al.*, 2007
AB433851	Samara_68/CG/2005	Kariwa *et al.*, 2009
KJ857340	Fuyuan-Mm-250	Wang *et al.*, 2014
KJ857338	Fuyuan-Mm-217	Wang *et al.*, 2014
KJ857331	Yakeshi-Mm-31	Wang *et al.*, 2014
KJ857330	Fuyuan-Mm-312	Wang *et al.*, 2014
KJ857329	Fuyuan-Mm-254	Wang *et al.*, 2014
AB676848	Kitahiyama128M/2008	Sanada *et al.*, 2012
AB675454	Sakhalin99M/1998	Kariwa *et al.*, 2012
AB675451	Tobetsu35M/2010	Kariwa *et al.*, 2012
AJ011647	Ls136V	Vapalahti *et al.*, 1999
KJ857333	Yuanjiang-Mf-13	Wang *et al.*, 2014
EU072488	Fusong-Mf-682	Zou *et al*., 2008
AJ011648	MF-43	Vapalahti *et al.*, 1999
EF422372	Fusong 84-05	Tang *et al.*, 2007
DQ518235	Bavaria CG 34/04	Essbauer *et al.*, 2007
DQ518220	Bavaria CG 151/05	Essbauer *et al.*, 2007
DQ518232	Bavaria CG 157/05	Essbauer *et al.*, 2007
DQ518234	Bavaria CG 159/05	Essbauer *et al.*, 2007
EU072489	Yakeshi-Mm-59	Zou *et al*., 2008
L08754	K27	Xiao *et al.*, 1993
L08755	P360	Xiao *et al.*, 1993
M29979	CG1820	Giebel *et al.*, 1989
X61034	Sotkamo	Vapalahti *et al.*, 1992
AB433854	Samara_128/CG/2005	Kariwa *et al.*, 2009

Table 13: continuation

Acc. No.	Strain (Isolate)	Reference
S segment tree DOBV		
KU529946	Sochi/hu	Krautkramer *et al.*, 2016
KT315642	Igneada/89Af/2009	Polat *et al.*, 2015
KT315640	Igneada/57Af/2009	Polat *et al.*, 2015
KT315638	Igneada/13Af/2009	Polat *et al.*, 2015
KT315637	Igneada/09Af/2009	Polat *et al.*, 2015
KF425494	ST-RS3	Stamenkovic *et al.*, 2013
KF425493	ST-RS1	Stamenkovic *et al.*, 2013
JQ344114	GER/10/1206/Aa	Rasche *et al.*, 2013
JQ026204	GER/08/131/Af	Popugaeva *et al.*, 2012
GU904029	Slo/Af-BER	Klempa *et al.*, 2010
GQ205408	GER/08/131/Af	Schlegel *et al.*, 2009
GQ205407	GER/08/118/Aa	Schlegel *et al.*, 2009
GQ205406	GER/05/477/Af	Schlegel *et al.*, 2009
GQ205405	GER/05/239/Aa	Schlegel *et al.*, 2009
GQ205404	GER/07/1064/Aa	Schlegel *et al.*, 2009
GQ205403	GER/07/424/Aa	Schlegel *et al.*, 2009
GQ205402	GER/07/607/Af	Schlegel *et al.*, 2009
GQ205401	GER/07/293/Aa	Schlegel *et al.*, 2009
HQ174470	Kardjali/2010	Papa & Christova, 2011
HQ174469	Smolyan/2010	Papa & Christova, 2011
HQ174468	Blagoevgrad/2009	Papa & Christova, 2011
FJ986109	Beskydy-08	Papa *et al.*, 2010
JF499666	DOBcro231lm	Markotic *et al.*, 2002
GQ205398	Lip17/hu	Dzagurova *et al.*, 2009
GQ205397	Lip7/hu	Dzagurova *et al.*, 2009
GQ205396	Lip6/hu	Dzagurova *et al.*, 2009
GQ205395	Lip5/hu	Dzagurova *et al.*, 2009
GQ205394	Lip4/hu	Dzagurova *et al.*, 2009
GQ205393	Lip2/hu	Dzagurova *et al.*, 2009
KT971014	Geshtenjas/Pogradec/261Af	Papa *et al.*, 2016
KT971013	Mollas/Kolonje/243Af	Papa *et al.*, 2016
KT971012	Dardhe/Korce/219Af	Papa *et al.*, 2016
KT971011	Diellas/Korce/201Af	Papa *et al.*, 2016
KT971010	Llogora/Vlore/85Af	Papa *et al.*, 2016
KT971009	Voskopoje/Korce/65Af	Papa *et al.*, 2016
KT971008	Togez/Librazhd/54Af	Papa *et al.*, 2016
KT971007	Llasen/Diber/22Af	Papa *et al.*, 2016

Table 13: continuation

Acc. No.	Strain (Isolate)	Reference
S segment tree DOBV		
KT971006	Pilafe/Diber/20Af	Papa *et al.*, 2016
KT971005	Brezhdan/Diber/19Af	Papa *et al.*, 2016
KT315641	Igneada/84Af/2009	Polat *et al.*, 2015
KT315639	Igneada/32Af/2009	Polat *et al.*, 2015
KT315636	Igneada/03Af/2009	Polat *et al.*, 2015
KT315635	Igneada/02Af/2009	Polat *et al.*, 2015
KP878313	10752/hu	Kruger *et al.*, 2015
KP878312	10645/Ap	Kruger *et al.*, 2015
KP878311	10636/Ap	Kruger *et al.*, 2015
KT885043	Stamforrad/POR	Miles & Lewandowski, 2015
KF776882	HtSi_1012_a1997	Korva *et al.*, 2013
KF776853	HtSi_1091_a1997	Korva *et al.*, 2013
KF776852	HtSi_1096_a1995	Korva *et al.*, 2013
KF776851	HtSi_273_p2008	Korva *et al.*, 2013
KF776850	HtSi_286_p2009	Korva *et al.*, 2013
KF776849	HtSi_274_p2008	Korva *et al.*, 2013
KF776848	HtSi_297_p2010	Korva *et al.*, 2013
KF776847	HtSi_299_p2010	Korva *et al.*, 2013
KF776846	HtSi_301_p2010	Korva *et al.*, 2013
KF776845	HtSi_275_p2008	Korva *et al.*, 2013
KF776844	HtSi_276_p2008	Korva *et al.*, 2013
KF776843	HtSi_268_p2008	Korva *et al.*, 2013
KF776842	HtSi_279_p2008	Korva *et al.*, 2013
KF776841	HtSi_261_p2008	Korva *et al.*, 2013
KF776840	HtSi_239_p2008	Korva *et al.*, 2013
KF776839	HtSi_256_p2008	Korva *et al.*, 2013
KF776838	HtSi_231_p2007	Korva *et al.*, 2013
KF776837	HtSi_230_p2007	Korva *et al.*, 2013
KF776836	HtSi_215_p2005	Korva *et al.*, 2013
KF776835	HtSi_207_p2005	Korva *et al.*, 2013
KF776834	HtSi_200_p2005	Korva *et al.*, 2013
KF776833	HtSi_129_p2000	Korva *et al.*, 2013
KF776832	HtSi_132_p2000	Korva *et al.*, 2013
KF776831	HtSi_136_p2001	Korva *et al.*, 2013
KF776830	HtSi_193_p2005	Korva *et al.*, 2013
KF776829	HtSi_1022_a2000	Korva *et al.*, 2013
KF776828	HtSi_1030_a1995	Korva *et al.*, 2013

Table 13: continuation

Acc. No.	Strain (Isolate)	Reference
S segment tree DOBV		
KF776827	HtSi_1038_a2002	Korva *et al.*, 2013
KF776826	HtSi_130_p2000	Korva *et al.*, 2013
KF776825	HtSi_1041_a2005	Korva *et al.*, 2013
KF776824	HtSi_1040_a1999	Korva *et al.*, 2013
KF776823	HtSi_1043_a1995	Korva *et al.*, 2013
KF776822	HtSi_1095_a1997	Korva *et al.*, 2013
KF776821	HtSi_1042_a2008	Korva *et al.*, 2013
KF776820	HtSi_1037_a2001	Korva *et al.*, 2013
KF776819	HtSi_1036_a2001	Korva *et al.*, 2013
KF776818	HtSi_1035_a2002	Korva *et al.*, 2013
KF776817	HtSi_1034_a2001	Korva *et al.*, 2013
KF776816	HtSi_1033_a2001	Korva *et al.*, 2013
KF776815	HtSi_1032_a2001	Korva *et al.*, 2013
KF776814	HtSi_1031_a2001	Korva *et al.*, 2013
KF776813	HtSi_1029_a1995	Korva *et al.*, 2013
KF776812	HtSi_1024_a2000	Korva *et al.*, 2013
KF776811	HtSi_1021_a1995	Korva *et al.*, 2013
KF776810	HtSi_1020_a2008	Korva *et al.*, 2013
KF776809	HtSi_1018_a2007	Korva *et al.*, 2013
KF776808	HtSi_1015_a1995	Korva *et al.*, 2013
KF776806	HtSi_1010_a2007	Korva *et al.*, 2013
KF776807	HtSi_1011_a2007	Korva *et al.*, 2013
KF776805	HtSi_1009_a1995	Korva *et al.*, 2013
KF776804	HtSi_1008_a1999	Korva *et al.*, 2013
KF776803	HtSi_1013_a2002	Korva *et al.*, 2013
KF776802	HtSi_1090_a2000	Korva *et al.*, 2013
KF776801	HtSi_1092_a1997	Korva *et al.*, 2013
KF776800	HtSi_1097_a1995	Korva *et al.*, 2013
KF776799	HtSi_1014_a2002	Korva *et al.*, 2013
KF776798	HtSi_1019_a2007	Korva *et al.*, 2013
KF776797	HtSi_270_p2008	Korva *et al.*, 2013
KF776796	HtSi_499_p2012	Korva *et al.*, 2013
KF776795	HtSi_492_p2012	Korva *et al.*, 2013
KF776794	HtSi_465_p2012	Korva *et al.*, 2013
KF776793	HtSi_397_p2012	Korva *et al.*, 2013
KC676608	As825/2007	Svoboda *et al.*, 2014
KC676607	As824/2007	Svoboda *et al.*, 2014

Table 13: continuation

Acc. No.	Strain (Isolate)	Reference
S segment tree DOBV		
KC676606	As822/2007	Svoboda *et al.*, 2014
KC676605	As820/2007	Svoboda *et al.*, 2014
KC676604	Af814/2007	Svoboda *et al.*, 2014
KC676603	Af815/2007	Svoboda *et al.*, 2014
KC676602	Af813/2007	Svoboda *et al.*, 2014
KC676601	Af970/2008	Svoboda *et al.*, 2014
KC676600	Af968/2008	Svoboda *et al.*, 2014
KC676599	Af967/2008	Svoboda *et al.*, 2014
KC676598	Af966/2008	Svoboda *et al.*, 2014
KC676597	Af965/2008	Svoboda *et al.*, 2014
KC676596	Af964/2008	Svoboda *et al.*, 2014
KC676595	Af957/2008	Svoboda *et al.*, 2014
KC676594	Af956/2008	Svoboda *et al.*, 2014
KC676593	Af933/2008	Svoboda *et al.*, 2014
KC676592	Af910/2008	Svoboda *et al.*, 2014
KC676591	Af903/2008	Svoboda *et al.*, 2014
KC676590	Af894/2008	Svoboda *et al.*, 2014
KC676589	Af882/2008	Svoboda *et al.*, 2014
KJ154958	KOS-1	Emmerich *et al.*, 2014
KF039739	Istanbul/H56/10	Ettinger *et al.*, 2013
FN377828	Af27/HungaryTR17/00	Plyusnina *et al.*, 2009
KC848501	Sarmellek/77Aa/06	Nemeth, 2013
KC848500	DOB/Pecs/31Aa/06	Nemeth, 2013
KC848499	DOB/Pecs/27Aa/06	Nemeth, 2013
KC848498	DOB/Gola/343Af/07	Nemeth, 2013
KC848497	DOB/Gola/291Af/07	Nemeth, 2013
KC848496	DOB/Gola/242Af/07	Nemeth, 2013
KC848495	DOB/Gola/235Af/07	Nemeth, 2013
KC848494	DOB/Pecs/210Af/07	Nemeth, 2013
JF920152	Ap/Sochi/79	Dzagurova *et al.*, 2012
JF920151	Ap/Sochi/43	Dzagurova *et al.*, 2012
JF920150	Ap/Sochi/hu	Dzagurova *et al.*, 2012
FN813292	Af56/2003	Plyusnina *et al.*, 2011
L41916	3970/87	Avsic-Zupanc *et al.*, 1995
EU188452	Aa1854/Lipetsk-02	Klempa *et al.*, 2008
EU188449	Ap1584/Sochi-01	Klempa *et al.*, 2008
EU562991	OMSK/180_Ap_ag/05	Garanina *et al.*, 2009

Table 13: continuation

Acc. No.	Strain (Isolate)	Reference
S segment tree DOBV		
EU562990	OMSK/189_Ap_ag/05	Garanina *et al.*, 2009
EU562989	OMSK/172_Ap_ag/05	Garanina *et al.*, 2009
AF442623	P-s1223/Krasnodar-2000	Dekonenko *et al.*, 2016
AF442622	Ap-1/Goryachiy Klyuch-2000	Dekonenko *et al.*, 2016
DQ305281	DOB/PV/02	Papa *et al.*, 2006
DQ305280	DOB/TV/02	Papa *et al.*, 2006
DQ305279	DOB/MD/02	Papa *et al.*, 2006
AF039525	ASA	Papa *et al.*, 2016
NC_005233	Ano-Poroia/Afl9/1999	Nemirov *et al.*, 2003
AJ410615	Ano-Poroia/Afl9/1999	Nemirov *et al.*, 2003
AJ410619	Ano-Poroia/13Af/99	Nemirov *et al.*, 2003
AJ131673	Kurkino/53Aa/98	Plyusnin *et al.*, 1999
AJ131672	Kurkino/44Aa/98	Plyusnin *et al.*, 1999
AJ269554	East Slovakia-400-Af	Sibold *et al.*, 2001
AJ269550	East Slovakia-862-Aa	Sibold *et al.*, 2001
AJ269549	East Slovakia-856-Aa	Sibold *et al.*, 2001
AJ251997	Kocevje/Af-3/93	Avsic-Zupanc *et al.*, 2000
AJ251996	Dobrava/Af-1/90	Avsic-Zupanc *et al.*, 2000
AJ009775	Saaremaa/90Aa/97	Nemirov *et al.*, 1999
AJ009773	DOB/Saaremaa/160V	Nemirov *et al.*, 1999
AF060024	DOB-PA	Papa *et al.*, 1998
AF060023	DOB-TD	Papa *et al.*, 1998
AF060022	DOB-SZ	Papa *et al.*, 1998
AF060021	DOB-HA	Papa *et al.*, 1998
AF060020	DOB-EA	Papa *et al.*, 1998
AF060019	DOB-GA	Papa *et al.*, 1998
AF060018	DOB-PR	Papa *et al.*, 1998
AF060017	DOB-SI	Papa *et al.*, 1998
AF060016	DOB-CG	Papa *et al.*, 1998
AF060015	DOB-TI	Papa *et al.*, 1998
AF060014	DOB-NF	Papa *et al.*, 1998
AY533121	Esl/197Aa/01	Klempa *et al.*, 2004
AY533119	Esl/33Aa/01	Klempa *et al.*, 2004
AY533117	H169/02	Klempa *et al.*, 2004
AY168576	East Slovakia/400Af/98	Klempa *et al.*, 2004
AY533120	Esl/81Aa/01	Klempa *et al.*, 2004
AY533118	Esl/29Aa/01	Klempa *et al.*, 2004

Table 13: continuation

Acc. No.	Strain (Isolate)	Reference
S segment tree DOBV		
AY961615	SK/Aa	Klempa *et al.*, 2004
AY961618	Esl/34Aa/01	Klempa *et al.*, 2004
HQ834507	P09072	Ma *et al.*, 2012
HQ834506	H10150	Ma *et al.*, 2012
HQ834505	CA10081905	Ma *et al.*, 2012
AF288646	A16	Yao *et al.*, 2001
EF990914	CGAa4P15	Zou *et al.*, 2008
EU363813	CGHu2	Zou *et al.*, 2008
AF288294	LR1	Yao *et al.*, 2000
EF990907	CGRn53	Zou *et al.*, 2008
EF208929	CJAp93	Zhang *et al.*, 2007
AF288659	S85-46	Yao *et al.*, 2001
EU092221	CGRn45	Zou *et al.*, 2008
JQ083395	HV004	Li *et al.*, 2012
U37768	CUMC-B11	Kim & Jung, 1995
KU207209	US8A15-1	Kim *et al.*, 2016
KU207207	ROKA14-11	Kim *et al.*, 2016
KU207197	Aa15-59	Kim *et al.*, 2016
KU207196	Aa15-58	Kim *et al.*, 2016
KU207195	Aa15-56	Kim *et al.*, 2016
KU207194	Aa10-265	Kim *et al.*, 2016
KU207193	Aa09-410	Kim *et al.*, 2016
KU207192	Aa09-17	Kim *et al.*, 2016
KU207191	Aa05-246	Kim *et al.*, 2016
KU207206	ROKA13-8	Kim *et al.*, 2016
KU207190	Aa04-722	Kim *et al.*, 2016
KT935057	Aa14-438	Song & Kim, 2016
KT935056	Aa14-423	Song & Kim, 2016
KT935055	Aa14-412	Song & Kim, 2016
KT935029	Aa05-249	Song & Kim, 2016
KT885049	76-118/POR	Miles & Lewandowski, 2015
GU329991	YN509	Cao *et al.*, 2010
AF321094	Maaji-1	Choi *et al.*, 2001
AF321095	Maaji-2	Choi *et al.*, 2001
AF427323	AP1168	Dekonenko *et al.*, 2001
AF427318	AA1028	Dekonenko *et al.*, 2001
KP970583	JS12	Wang, 2016

Table 13: continuation

Acc. No.	Strain (Isolate)	Reference
S segment tree DOBV		
KJ857348	Fuyuan-Aa-36	Wang *et al.*, 2014
KJ857347	Fuyuan-Aa-26	Wang *et al.*, 2014
KC570390	DandongHu-91	Cheng *et al.*, 2014
KC490914	Rr/MDG/2009/ATD261	Reynes *et al.*, 2014
KC490915	Rr/MDG/2009/ATD9	Reynes *et al.*, 2014
KC490916	Rr/MDG/2009/ATD56	Reynes *et al.*, 2014
KC490917	Rr/MDG/2009/ATD108	Reynes *et al.*, 2014
KC490918	Em/MDG/2009/ATD49	Reynes *et al.*, 2014
JQ082300	SA14	Klempa *et al.*, 2012
JQ082303	SA22	Klempa *et al.*, 2012
AB618112	CSG5	Luan *et al.*, 2012
AF329389	Tchoupitoulas (TCH)	Shi *et al.*, 2003
AF329388	IR461	Shi *et al.*, 2003
KP645198	Fj372/2013	Wang *et al.*, 2015
JX853575	DPRK08	Yao *et al.*, 2012
KU204960	Tchoupitoulas/POR	Miles *et al.*, 2016
KF387725	LYON/Rn/FRA/2013/LYO852	Dupinay *et al.*, 2014
JX879769	Humber	Jameson *et al.*, 2013
KP859512	JiangxiXinjianRn-09-2011	Liu *et al.*, 2015
KP859511	JiangxiXinjianRn-07-2011	Liu *et al.*, 2015
GU592951	ShenyangRn139	Guo *et al.*, 2011
GU592950	ShenyangRn19	Guo *et al.*, 2011
GU592953	RuianRn180	Guo *et al.*, 2011
JQ898106	SEO/Belgium/Rn895/2005	Plyusnina *et al.*, 2012
GU592948	GuangzhouRn36	Guo *et al.*, 2011
GU592946	Longwan581	Guo *et al.*, 2011
GU592945	RuianRn242	Guo *et al.*, 2011
GU592944	XiaotangshanRn7	Guo *et al.*, 2011
GU592942	FeixianRn1	Guo *et al.*, 2011
GU592941	GaomiRn9	Guo *et al.*, 2011
GU592940	JiningCt13	Guo *et al.*, 2011
GU592938	GaomiRn47	Guo *et al.*, 2011
GU592937	JinanRn1	Guo *et al.*, 2011
GU592936	HebeiRn9	Guo *et al.*, 2011
GU592933	GanyuRn137	Guo *et al.*, 2011
GU361893	SC106	Chen *et al.*, 2010
FJ803217	RuianRr57	Lin *et al.*, 2016

Table 13: continuation

Acc. No.	Strain (Isolate)	Reference
S segment tree DOBV		
FJ803208	OuhaiRf35	Lin *et al.*, 2016
FJ803206	CixiRn76	Lin *et al.*, 2016
KF745951	Taonan420	Fan *et al.*, 2014
JQ665928	WuhanRn98	Kang *et al.*, 2012
GQ274945	Singapore/06(RN46)	Johansson *et al.*, 2010
KJ950869	Seoul-Baxter/NYC-D3_S	Firth *et al.*, 2014
KX079476	IR162	Lloyd *et al.*, 1984
KX079473	IR33	Lloyd *et al.*, 1984
M segment tree DOBV		
JQ026205	GRW/Aa	Popugaeva *et al.*, 2012
GQ205413	GER/08/131/Af	Schlegel *et al.*, 2009
GQ205412	GER/08/118/Aa	Schlegel *et al.*, 2009
GQ205411	GER/05/477/Af	Schlegel *et al.*, 2009
GQ205410	GER/07/607/Af	Schlegel *et al.*, 2009
GQ205409	GER/07/293/Aa	Schlegel *et al.*, 2009
GQ205400	Lip9/hu	Dzagurova *et al.*, 2009
GQ205399	Lip2/hu	Dzagurova *et al.*, 2009
KT885042	Stamforrad/POR	Miles & Lewandowski, 2015
KF776791	HtSi_297_p2010	Korva *et al.*, 2013
KF776790	HtSi_301_p2010	Korva *et al.*, 2013
KF776789	HtSi_299_p2010	Korva *et al.*, 2013
KF776788	HtSi_286_p2009	Korva *et al.*, 2013
KF776787	HtSi_279_p2008	Korva *et al.*, 2013
KF776786	HtSi_276_p2008	Korva *et al.*, 2013
KF776785	HtSi_275_p2008	Korva *et al.*, 2013
KF776784	HtSi_270_p2008	Korva *et al.*, 2013
KF776783	HtSi_268_p2008	Korva *et al.*, 2013
KF776782	HtSi_256_p2008	Korva *et al.*, 2013
KF776780	HtSi_231_p2007	Korva *et al.*, 2013
KF776779	HtSi_230_p2007	Korva *et al.*, 2013
KF776778	HtSi_215_p2005	Korva *et al.*, 2013
KF776777	HtSi_207_p2005	Korva *et al.*, 2013
KF776776	HtSi_193_p2005	Korva *et al.*, 2013
KF776775	HtSi_135_p2001	Korva *et al.*, 2013
KF776774	HtSi_132_p2000	Korva *et al.*, 2013
KF776773	HtSi_130_p2000	Korva *et al.*, 2013
KF776769	HtSi_1009_a1995	Korva *et al.*, 2013

Table 13: continuation

Acc. No.	Strain (Isolate)	Reference
M segment tree DOBV		
KF776768	HtSi_1043_a1995	Korva *et al.*, 2013
KF776767	HtSi_1015_a1995	Korva *et al.*, 2013
KF776766	HtSi_1014_a2002	Korva *et al.*, 2013
KF776765	HtSi_1013_a2002	Korva *et al.*, 2013
KF776763	HtSi_1039_a1996	Korva *et al.*, 2013
KF776762	HtSi_1032_a2001	Korva *et al.*, 2013
KF776745	HtSi_1038_a2002	Korva *et al.*, 2013
KF776746	HtSi_1035_a2002	Korva *et al.*, 2013
KF776744	HtSi_1031_a2001	Korva *et al.*, 2013
KF776742	HtSi_1022_a2000	Korva *et al.*, 2013
KF776740	HtSi_1018_a2007	Korva *et al.*, 2013
KF776738	HtSi_1010_a2007	Korva *et al.*, 2013
KF776736	HtSi_1008_a1999	Korva *et al.*, 2013
KC676628	Af967/2008	Svoboda *et al.*, 2014
KC676627	Af970/2008	Svoboda *et al.*, 2014
KC676626	Af968/2008	Svoboda *et al.*, 2014
KC676625	Af966/2008	Svoboda *et al.*, 2014
KC676624	Af965/2008	Svoboda *et al.*, 2014
KC676623	Af964/2008	Svoboda *et al.*, 2014
KC676622	Af957/2008	Svoboda *et al.*, 2014
KC676621	Af956/2008	Svoboda *et al.*, 2014
KC676619	Af910/2008	Svoboda *et al.*, 2014
KC676620	Af933/2008	Svoboda *et al.*, 2014
KC676618	Af903/2008	Svoboda *et al.*, 2014
GU904035	Slo/Af-BER	Kirsanovs *et al.*, 2010
JF920149	Ap/Sochi/hu	Dzagurova *et al.*, 2012
L33685	Dobrava	Avsic-Zupanc *et al.*, 1995
EU188453	Aa1854/Lipetsk-02	Klempa *et al.*, 2008
EU188450	Ap1584/Sochi-01	Klempa *et al.*, 2008
NC_005234	Ano-Poroia/Afl9/1999	Nemirov *et al.*, 2003
AJ410616	Ano-Poroia/Afl9/1999	Nemirov *et al.*, 2003
AJ009774	DOB/Saaremaa/160V	Nemirov *et al.*, 1999
AY168578	East Slovakia/862Aa/97	Klempa *et al.*, 2003
AY168577	East Slovakia/400Af/98	Klempa *et al.*, 2003
AY961616	SK/Aa	Klempa *et al.*, 2005
KC490921	Rr/MDG/2009/ATD56	Reynes *et al.*, 2014
KC490919	Em/MDG/2009/ATD49	Reynes *et al.*, 2014

Table 13: continuation

Acc. No.	Strain (Isolate)	Reference
M segment tree DOBV		
KC490920	Rr/MDG/2009/ATD261	Reynes *et al.*, 2014
JQ082301	SA14	Klempa *et al.*, 2012
DQ371905	Q32	Dai *et al.*, 2006
AF288645	A16	Yao *et al.*, 2001
EU363819	CGHu2	Zou *et al.*, 2008
EF990921	CGRn53	Zou *et al.*, 2008
AF288658	S85-46	Yao *et al.*, 2001
AF288656	SN7	Yao *et al.*, 2001
JQ083394	HV004	Li *et al.*, 2012
KU207203	ROKA14-11	Kim *et al.*, 2016
KU207202	ROKA13-8	Kim *et al.*, 2016
KT935015	Aa14-362	Song & Kim, 2016
KT935010	Aa14-198	Song & Kim, 2016
KT935006	Aa10-561	Song & Kim, 2016
KT935003	Aa10-288	Song & Kim, 2016
KT934999	Aa09-652	Song & Kim, 2016
KT934996	Aa05-331	Song & Kim, 2016
GQ120966	Z251	Yao *et al.*, 2009
D25529	cl-1	Isegawa *et al.*, 1994
KJ857336	Fuyuan-Aa-145	Wang *et al.*, 2014
JQ912875	LongquanMf-09-8	Wang *et al.*, 2013
JQ912859	LongquanAa-10-393	Wang *et al.*, 2013
JQ912850	LongquanAa-09-541	Wang *et al.*, 2013
JQ912835	LongquanAa-09-350	Wang *et al.*, 2013
JQ912829	LongquanAa-09-24	Wang *et al.*, 2013
JQ912810	LongquanAa-08-235	Wang *et al.*, 2013
AB620032	Galkino/AA57/2002	Kariwa *et al.*, 2012
FJ753399	ZLS-12	Yao *et al.*, 2009
AB127995	Bao14	Lokugamage *et al.*, 2004
KX687231	Aa14_239	Song *et al.*, 2016
AB618130	5CSG	Luan *et al.*, 2012
KU207166	Aa14-204	Song *et al.*, 2016
S47716	80-39	Antic *et al.*, 1991
AF288654	K24-v2	Yao *et al.*, 2001
AF288652	K24-e7	Yao *et al.*, 2001
AF288650	Gou3-e5	Yao *et al.*, 2001
AF288298	L99	Zhihui *et al.*, 2000

Table 13: continuation

Acc. No.	Strain (Isolate)	Reference
M segment tree DOBV		
AF145977	Gou3	Yao *et al.*, 1999
KP645197	Fj372/2013	Wang *et al.*, 2015
JX853576	DPRK08	Yao *et al.*, 2012
KU204959	Tchoupitoulas/POR	Miles *et al.*, 2016
KF387724	LYON/Rn/FRA/2013/LYO852	Dupinay *et al.*, 2014
JX879768	Humber	Jameson *et al.*, 2012
KM948597	Humber	Jameson *et al.*, 2013
KP859513	JiangxiXinjianRn-07-2011	Liu *et al.*, 2015
KP859514	JiangxiXinjianRn-09-2011	Liu *et al.*, 2015
KM948593	Cherwell	Taori *et al.*, 2013
KM233660	HBT50	Li & Cai, 2014
KM233658	HBT14	Li & Cai, 2014
KM233659	HBT49	Li & Cai, 2014
KM233657	HBQ43	Li & Cai, 2014
KM233656	HBQ20	Li & Cai, 2014
KM233655	HBQ17	Li & Cai, 2014
KM233654	HBQ7	Li & Cai, 2014
KM233653	HBQ5	Li & Cai, 2014
KM115585	93HBQ3	Li & Cai, 2014
NC_005237	80-39	Antic *et al.*, 1991
EU163437	China	Zhang *et al.*, 2007
D17594	KI-88-15	Kariwa *et al.*, 1994
D17593	KI-85-1	Kariwa *et al.*, 1994
D17592	KI-83-262	Kariwa *et al.*, 1994

APPENDIX 3

Detailed parameter descriptions of phylogenetic trees

Figure 12: Phylogenetic tree constructed from the partial L segment sequences of 226nt. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura 3-parameter model (Tamura, 1992). The tree with the highest log likelihood (-6608.3713) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 0.7706)). The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 17.6339% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

Figure 13: Phylogenetic tree constructed from the partial S segment sequences of 501nt. The evolutionary history was inferred by using the Maximum Likelihood method based on the GTR model chosen according to BIC (Nei and Kumar, 2000). The tree description is equivalent as in the Figure 12 with the parameters:

- the highest log likelihood (-15180.1275) is shown.
- bootstrap statistical support repeated 1000 times, values $\geq$ 70% are shown.
- a discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 0.5568)).
- evolutionarily invariable ([+*I*], 21.3265% sites).
- 324 nucleotide sequences involved.

Figure 14: Phylogenetic tree constructed from the partial M segment sequences of 576nt. The evolutionary history was inferred by using the Maximum Likelihood method

based on the Tamura-Nei model chosen according to BIC (Tamura and Nei, 1993). The tree description is equivalent as in the Figure 12 with the parameters:

- the highest log likelihood (-7636.2282) is shown.
- bootstrap statistical support repeated 1000 times, values ≥ 70% are shown.
- a discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 0.6312)).
- evolutionarily invariable ([+*I*], 25.3054% sites).
- 104 nucleotide sequences involved.

Compressed clade marked as * includes following sequences: T4_10_087, T4_10_203, T4_10_081, T4_10_080, T4_10_049, T4_10_043, T4_10_041, T4_10_032, T4_10_030, T4_10_026, T4_10_022, T4_10_020, T4_10_014, T4_10_012, T4_10_006, T4_10_019, DQ518237, T2_10_006, T2_10_046, T2_10_048, T2_10_063, T2_10_076, T2_10_087, T2_10_103.

Figure 15: Phylogenetic tree constructed from the partial S segment sequences of 519nt. The evolutionary history was inferred by using Maximum Likelihood method based on Tamura 3-parameter model chosen according to BIC (Tamura, 1992). The tree description is equivalent as in the Figure 12 with the parameters:

- the highest log likelihood (-10744.1993) is shown.
- bootstrap statistical support repeated 1000 times, values ≥ 70% are shown.
- a discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 0.6250)).
- evolutionarily invariable ([+*I*], 22.1248% sites).
- 268 nucleotide sequences involved.

Figure 16: Phylogenetic tree constructed from the partial M segment sequences of 456nt. The evolutionary history was inferred by using Maximum Likelihood method based on Tamura 3-parameter model chosen according to BIC (Tamura, 1992). The tree description is equivalent as in the Figure 12 with the parameters:

- the highest log likelihood (-6941.0853) is shown.
- bootstrap statistical support repeated 1000 times, values ≥ 70% are shown.

- a discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 0.6682)).
- evolutionarily invariable ([+*I*], 23.5746 % sites).
- 127 nucleotide sequences involved.