HELENA SIROLA

Serological Rapid Tests for Detection of Human and Rodent Hantavirus Infections

Doctoral dissertation

To be presented by permission of the Faculty of Natural and Environmental Sciences of the University of Kuopio for public examination in Auditorium L 21, University of Kuopio, Friday 5th September 2003, at 12 noon

Department of Chemistry

University of Kuopio

ABSTRACT

Hantaviruses belonging to the family Bunyaviridae, constitute a genus with over 20 different viruses. The most prevailing pathogenic hantaviruses in Eurasia are Hantaan (HTNV), Puumala (PUUV), Dobrava (DOBV), Seoul, and Saaremaa viruses, which cause hemorrhagic fever with renal syndrome in humans. The severity of the disease varies from mild to life-threatening, and hospitalization is needed in a number of cases. Early and correct diagnosis is a prerequisite for successful treatment and improved recovery rates from hantavirus infections. Thus more efficient diagnostic tools and increasing awareness of hantavirus infections are needed on global basis.

The aim of this study was to develop more swift diagnostic tools for hantavirus infections. New serological immunochromatographic rapid tests for detection of acute PUUV, DOBV, and HTNV infections in humans, and for detection of hantavirus-specific IgG antibodies in rodents were introduced in this thesis. The rapid tests use recombinant, full-length nucleocapsid proteins of PUUV, DOBV and HTNV as antigens. The N proteins were produced in insect cell cultures with baculovirus vectors, extracted with urea or guanidine hydrochloride, and purified with affinity chromatography. The diagnostic potential of the N proteins was found to be adequate compared to the conventional EIA method. The proteins adapted well to the lateral-flow immunochromatographic rapid test format, and the developed method showed good diagnostic value in hantavirus diagnostics. The rapid tests were user-friendly and their diagnostic performances were comparable to conventional home-made and commercially available EIA and IFA methods. The performances of the human tests were adequate especially with unfrozen serum samples, and old immunity to hantaviruses or other viral infections did not interfere notably with the rapid tests. Furthermore, the rodent IgG rapid test was found to be an efficient and convenient method for screening of hantavirus antibodies among bank vole and other rodent populations in laboratory and field conditions.

Serological cross-reactions of the rapid tests/antigens were studied with human and rodent samples. The PUUV antigen showed adequate performance among closely related hantaviruses carried in the arvicoline host clade, when the PUUV-IgG rapid tests for rodents were assayed with samples containing IgG antibodies to Topografov and Tula hantaviruses. Nonetheless, it was evident that the PUUV-N antigen did not provide enough serological cross-reactivity to hantaviruses carried by murine rodents (e.g. HTNV and DOBV). When the human PUUV-IgM test was assayed with DOBV and HTNV positive samples, cross reactions were uncommon. Additionally, it was noted that even though DOBV and HTNV cross-react with each other frequently, they do not cross-react sufficiently for diagnostic purposes. This supports the use of all three antigens in the detection of hantavirus antibodies in humans and also in rodents in the Eurasian continent. Studies to expand the rapid test technology to the hantaviruses carried by sigmodontine rodents are in progress (e.g. Sin Nombre virus). If the technology is successfully expanded to other animal and virus species, it might provide a useful tool to monitor and control the dynamics of emerging zoonotic viruses in host animal populations.

National Library of Medicine Classification: QW 168.5.B9, QY 265, QW 575
Medical Subject Headings: hantavirus; hantavirus infections / diagnosis; Puumala virus; Hantaan virus; hemorrhagic fever with renal syndrome; serologic tests; nucleocapsid proteins; recombinant proteins; antibodies; cross reactions; enzyme-linked immunosorbent assay; comparative study
To my loving husband, Joonas
“I only hope that we don’t lose sight of one thing - that it was all started by a mouse.”

-Walt Disney-
ACKNOWLEDGEMENTS

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Kuopio, August 2003

Helena Sirola
ABBREVIATIONS

Ab  antibody
Ag  antigen
AMRV  Amur virus
ANDV  Andes virus
ARAV  Araraquara virus
AVI  avidity detection of antibodies
BAYV  Bayou virus
BCCV  Black Creek Canal virus
BLLV  Bloodland lake virus
BRV  Blue River virus
BSA  bovine serum albumin
CADV  Cano Delgadito virus
CASV  Castelo dos Sonhos virus
CCHF  Crimean-Congo hemorrhagic fever
CDC  Centers for disease Control and Prevention
CF  complement fixation
CRP  C-reactive protein
DBSV  Da Bie Shan virus
CHOV  Cholo virus
DIC  disseminated intravascular coagulopathy
DOBV  Dobrava virus
DOBV-N  DOBV nucleocapsid protein
EIA  enzyme immunoassay
ELISA  enzyme linked immuno sorbent assay (means the same thing as EIA)
ELMCV  El Moro Canyon virus
FM  electron microscopy
ESR  erythrocyte sedimentation rate
FDA  U.S. Food and Drug Administration
FRNT  focus reduction neutralization test
G1  glycoprotein 1
G2  glycoprotein 2
hCG  human chorionic gonadotropin
HFRS  hemorrhagic fever with renal syndrome
HIV  human immunodeficiency virus
HPS  hantavirus pulmonary syndrome
IIRP  horseradish peroxidase
HTNV  Hantaan virus
HTNV-N  HTNV nucleocapsid protein
IFA  immuno-fluorescence assay
ICTV  International Committee on Taxonomy of Viruses
ISLAV  Isla Vista virus
KHAV  Khabarovsk virus
KHF  Korean hemorrhagic fever
LANV  Laguna Negra virus
<table>
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<td>LCR</td>
<td>ligase chain reaction</td>
</tr>
<tr>
<td>LECV</td>
<td>Lechiguana virus</td>
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<tr>
<td>LSCV</td>
<td>Limestone Canyon virus</td>
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<td>MACV</td>
<td>Maciel virus</td>
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<td>MGLV</td>
<td>Monongahela virus</td>
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<td>MULV</td>
<td>Muleshoe virus</td>
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<tr>
<td>N</td>
<td>nucleocapsid protein</td>
</tr>
<tr>
<td>NASBA</td>
<td>nucleic acid sequence-based amplification</td>
</tr>
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<td>NE</td>
<td>nephropathia epidemica</td>
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<td>NYV</td>
<td>New York virus</td>
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<tr>
<td>ORF</td>
<td>open reading frame</td>
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<tr>
<td>ORNV</td>
<td>Oran virus</td>
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<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PHV</td>
<td>Prospect Hill virus</td>
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<tr>
<td>PRGV</td>
<td>Pergamino virus</td>
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<tr>
<td>PUUV</td>
<td>Puumala virus</td>
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<tr>
<td>PUUV-N</td>
<td>PUUV nucleocapsid protein</td>
</tr>
<tr>
<td>RF</td>
<td>rheumatoid factor</td>
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<tr>
<td>RIOMV</td>
<td>Rio Mamore virus</td>
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<tr>
<td>RIOSV</td>
<td>Rio Segundo virus</td>
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<tr>
<td>RT</td>
<td>room temperature</td>
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<td>SAAV</td>
<td>Saaremaa virus</td>
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<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate – polyacrylamide gel electrophoresis</td>
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<td>Seoul virus</td>
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<td>SNV</td>
<td>Sin Nombre virus</td>
</tr>
<tr>
<td>THAIV</td>
<td>Thailand virus</td>
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<td>TOPV</td>
<td>Topografov virus</td>
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<td>TPMV</td>
<td>Thottapalayam virus</td>
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<td>Tula virus</td>
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<tr>
<td>TULV-N</td>
<td>TULV nucleocapsid protein</td>
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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on following publications referred to in the text by their Roman numerals. Some unpublished data are also presented.


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1 INTRODUCTORY REVIEW OF THE LITERATURE

1.1 History of virology

The beginning of the virology may be dated back to 1883 when the German scientist Adolph Mayer demonstrated that tobacco mosaic disease was contagious. He suggested that the infection was caused by a little bacterium, although this bacterium could not be cultured in the available media. In the 1890s, the Russian scientist Dimitri Ivanowsky demonstrated with the filtration method that the tobacco mosaic disease was caused by particles smaller than bacteria. He postulated that the disease was caused by bacterial toxins (69). The idea of viruses was primarily discovered by the Dutch scientist Martinus Beijerinck, who introduced the term “virus” in his articles (lat. Virus, poison/venom) at the end of the 1890s. He suggested that these viruses were in a liquid form and called them as “contagium vivum fluidum” (42). In 1897, foot-and-mouth disease was the first viral disease described in animals, and four years later the first human viral disease, yellow fever, was reported (68). Finally, 50 years after the original description of the causative agent of tobacco mosaic disease, the virus became “visible” when the American scientist Wendell Stanley crystallized tobacco mosaic virus in 1935. Since then, new viruses have been found frequently, and according to the latest report of the International Committee on Taxonomy of Viruses, there are more than 1550 virus species belonging to 3 orders, 56 families, 9 subfamilies and 233 genera recognized in the world (34).

1.2 Virus diagnostics: past and present

In the 1940s, virology was a new discipline of science and was not appreciated in the scientific world. Research centers were doing virus diagnostics only part-time and the diagnostics was mainly based on virus cultures in experimental animals or embryonated eggs, and the increase in antibodies by neutralization, hemagglutinin inhibition, or complement fixation tests. Major breakthroughs in virus diagnostics were seen in the
1950s when John Enders and his co-workers developed new and simpler cell culture methods, which made the culturing of viruses and the preparation of the viral antigens more convenient. John Enders together with Thomas Weller and Frederick Robbins received the Nobel Prize in 1954 for their discovery of the cultivation method for poliomyelitis viruses in various types of tissue cultures (24, http://www.nobel.se). In the 1950s, also the role of IgM antibodies was understood in the acute phase of viral infections, and the newly developed serological tests enabled the laboratory diagnosis from the sera of the acute illness (for a review see 68). A new, easy, and rapid serological method, the enzyme-linked immunosorbent assay (ELISA), was introduced in 1971 (35, 36, 37). In 1974, the method took a revolutionary step when it was performed on microwell plates (170) such that the test format became simple, easy, and cheap, and provided a diagnostic tool for simultaneous testing of hundreds of samples.

During the past decades, viral diagnostics have developed tremendously. Today virus isolation, antigen detection (EIA, IFA), nucleic acid detection (PCR, LCR, NASBA), electron microscopy (EM), and different serological methods (AVI, EIA, IFA, CF) are routinely used in laboratories, which are specialized for virus diagnostics (http://www.hus.fi). The serological confirmation of acute virus infection is done by detecting IgM antibodies, which are in most cases present only in the acute infection, or by detecting IgG antibodies with low avidity to antigen (AVI). The avidity of IgG antibodies to antigen increases considerably during the infection. Virus diagnostics are continuously becoming faster and more reliable than earlier with the introduction of new diagnostic tests. Although most of the diagnostics is made in centralized laboratories with semi-automated or automated equipment, there has been a tendency towards the development of more simple tests, which could be performed near the patient without any delay.

1.3 Overview of the commonly used rapid test technologies

The commonly used rapid test technologies may be roughly categorized into at least four groups: microplate / solid-phase, agglutination, flow-through, and lateral-flow
technologies (http://www.millipore.com, http://www.rapid-diagnostics.org, http://www.sartorius.de, http://www.whatman.com). Microplate assays are constituted by immobilizing the capture reagent on the non-porous solid-phase, which is usually made of polystyrene or glass. The samples are incubated on the solid-phase, washed, and incubated with the detector reagent. The signal can be generated by enzymatic or fluorescent reactions, or by binding latex or colloidal gold particles with a reporter molecule, e.g. a monoclonal antibody, a recombinant protein, or protein A.

The agglutination tests employ microscopic latex or gelatin beads, or preserved mammalian or avian blood cells, to which antigen or antibody is bound. The test is assayed on a microscope slide or in a microwell plate, in which the sample and the particles are added. The mixture is then stirred and allowed to settle. Formation of visible agglutination indicates a positive reaction.

The flow-through test includes a porous membrane through which the fluid flows into an absorbent pad, and a sub-membrane, that inhibits the back-flow of the liquid. The antigen or antibody is immobilized on the membrane, and the test is performed by pipeting sample, wash buffer, signal reagent and second wash buffer consecutively. These tests may use some enzyme, colored latex or colloidal gold particles as the signal transducer.

The lateral-flow tests are based on immunochromatography. The capture reagent is immobilized on the membrane and the signal reagent, which is usually colloidal gold, or a latex bead conjugate, is dried on its own pad. These tests are simple for the user to perform because only one or two steps, addition of sample and buffer together or separately are needed. The sample solubilizes the signal reagent and the liquid flows laterally through membrane and pads with the aid of capillary action.

1.3.1 Immunochromatographic test technology

Immunochromatographic test technology has been one of the most popular and convenient technologies for rapid detection of antigens or antibodies since its introduction in the 1980s. Patent applications covering the principles of the lateral-flow
immunochromatography were filed in the 1980s (25, 180), and soon after that the first tests entered the market. The first immunochromatographic rapid test was for the detection of human chorionic gonadotropin (hCG), i.e. the so-called pregnancy test, and for the first time it was possible to make a preliminary diagnosis at home or in a “non-professional” environment. Today immunochromatographic in vitro rapid tests exist for many purposes (some commercial tests being mentioned in parenthesis), e.g. screening for ovulation (OvuQuick One Step, Quidel Corporation, San Diego, California), for premature rupture of fetal membranes (Actim™PROM, Medix Biochemica, Kauniainen, Finland), for acute pancreatitis (Actim™ Pancreatitis, Medix Biochemica), for group A streptococcal antigen (28, 30, 173), and for Haemophilus ducreyi (137). Rapid tests for diagnosis of virus infections based either on antigen or antibody detection include e.g. the tests for diagnosis of dengue virus (Dengue Duo IgM & IgG Rapid Cassette test, PanBio, Columbia, Maryland) (84, 148, 169), human immunodeficiency virus (HIV) (146), influenza virus (QuickVue Influenza test, Quidel Corporation) (145), and hantavirus (I, III) infections. Furthermore, the rapid HIV test kit (OraQuick Rapid HIV-1 Antibody Test; OraSure Technologies Inc., Bethlehem, Pennsylvania) was approved as an official diagnostic tool for HIV in November 2002 by FDA (http://www.fda.gov). This action clearly demonstrates that the rapid tests have now gained a firm foothold in the field of virus diagnostics.

1.4 Hantaviruses

Hantaviruses constitute a genus in the family Bunyaviridae (149), which is the most diverse family of animal viruses. Hantaviruses are spherical, single-stranded RNA viruses with glycoprotein spikes projecting from the lipid envelope. They are about 80-110 nm in diameter (88), but also elongated virus particles (170 nm) can be formed. The negatively polarized hantavirus genome is divided into three segments, S, M and L, which encode the nucleocapsid protein (N), two envelope glycoproteins (G1 and G2) and viral RNA polymerase (L protein), respectively (Figure 1). The S segment of hantaviruses, carried by Arvicolinae and Sigmodontinae rodents, includes an additional open reading
frame (ORF), which codes probably a nonstructural protein termed NSs. Each genome segment is encapsulated by the nucleocapsid protein (139, 144).

![Schematic representation of a hantavirus particle.](image)

**Figure 1.** Schematic representation of a hantavirus particle.

The first known hantavirus, Hantaan virus (HTNV), was isolated in 1978 from the lung tissue of striped field mouse (*Apodemus agrarius*) (92). Hantaviruses are rodent-borne and as a general rule, each hantavirus is associated strictly with one specific rodent host species. This strict association reflects a classical example of virus-host co-evolution (143, 166). Hantaviruses are divided into three main groups on phylogenetic trees: HTNV-like viruses (HTNV, SEOV, DOBV, SAAV), PUUV-like viruses (PUUV, TULV, PHV, BLLV, ISLAV, TOPV, KHAV), and SNV-like viruses (SNV, NYV, BAYV, BCCV, LANV, ANDV, ELMCV, RIOSV, RIOMV). HTNV-like viruses are carried by *Murinae* rodents, which include mice and rats from the Old World. PUUV-like viruses are carried by voles and lemmings belonging to *Arvicolinae* rodents, and SNV-like viruses are carried by *Sigmodontinae* rodents, which include mice and voles from the New World. The N protein sequence homologies between hantavirus groups are 61-64% between PUUV and HTNV group, 61-65% between HTNV and SNV group, and 71-78% between
PUUV and SNV groups. Within individual groups, the N sequence homologies are over 79% (109, 144).

According to the VII report of the International Committee on Taxonomy of Viruses (ICTV), different hantavirus species 1) are found in a unique ecological niche, 2) exhibit at least a 7% difference in their amino acid composition in the complete G1, G2 and the N-protein, 3) show at least a fourfold difference in two-way cross-neutralization tests, and 4) do not naturally form reassortants with other species (34). However, the viruses that have undergone a host-switch event are usually more closely than 7% related in amino acid level with each other (for example NYV-SNV, KHAV-TOPV and DOBV-SAAV pairs). This has lead to the proposal that the strict 7% rule might not necessarily be needed in the demarcation in the hantavirus genus (143, 144). The latest report of ICTV declares that 23 virus species have been listed today in the genus *Hantavirus*. New hantavirus species emerge continuously, and several new hantaviruses have been described since publication of their most recent report (Table 1). Additionally, there are indications that yet unknown hantaviruses may circulate in Europe (107, 109).

### 1.5 Hantavirus infections in rodents

According to empirical studies, hantavirus infection in rodents seems to be asymptomatic. The infection does not seem to cause any harmful or abnormal effects on lifespan, e.g. survival or reproductive fitness of the infected rodents (7, 21, 115, 175). However, some histopathological changes have been associated in naturally or experimentally infected rodents. Mononuclear infiltrates, edematous alveolar septa, endothelial hyperplasia and evidence of inflammatory changes have been demonstrated in NYV, SNV or PUUV infected rodents (112).

PUUV is the most common hantavirus in Europe. It is prevalent in large regions of Eurasia and it causes a fairly mild disease in humans with low mortality. Thus PUUV serves as a model in hantavirus research. The transmission, viral shedding and antibody response studies described in chapters 1.5.1 and 1.5.2 were done mainly with bank voles infected with PUUV. The results obtained from PUUV studies may to some extent be applicable also to other hantaviruses.
Table 1. Hantaviruses, their abbreviations, rodent hosts, associated diseases, and distribution in the world. Official virus species names are underlined and the other names are either proposed or tentative. Hantaviruses that are currently known as human pathogens are typed in **bold**. (This table is adapted from 34, 91, 109, 143.)

<table>
<thead>
<tr>
<th>Virus species</th>
<th>Abbr.</th>
<th>Rodent host</th>
<th>Disease</th>
<th>Distribution</th>
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<tr>
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<td>AMRV</td>
<td>Apodemus peninsulae</td>
<td>HFRS</td>
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<tr>
<td>Andes</td>
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<td>Oligoryzomys longicaudatus</td>
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<td>DOBV</td>
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<td>FLMCV</td>
<td>Reithrodonomys megalotis</td>
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<tr>
<td>* Rossiev</td>
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<td><em>M. rossiev</em></td>
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* TULV is traditionally thought to be apathogenic to humans, but there is one reported case where TULV was suggested to evoke fever and exanthema in human (151).
1.5.1 Transmission among carrier rodents

Rodents are infected with hantaviruses horizontally from one individual to another, probably through absorption of aerosolized excreta, bites or scratches. The hantavirus infection is probably life-long, and the infected animal transmits the virus for the rest of its life. However, the highest transmission rates have been reported shortly after the infection, when the viral antigen is abundant in the tissues of the rodent (7, 175).

The infection rate in bank vole populations depends on the density, behavior, mobility and demographic factors (e.g. age, sex, sexual maturity, body mass index) of the population (7, 13, 39, 40, 130). Males, sexually active, old or heavy rodents are usually infected more often than young or female animals. Very young and newborn animals are protected from the infection up to three months by maternal antibodies (7, 26, 40, 49, 85, 179). After levels of maternal antibodies decline, the most probable ways of acquiring PUUV infection are behavioral contacts during mating, fights or communal nesting (40). Hantaviruses are very contagious within the rodent species they infect, and the rate of infection among carrier rodent populations fluctuates very much, e.g. depending on the population density, geographical area, season, food supply and predators. Thus, population dynamics of bank voles are quite different in various parts of Europe, but generally peak years of rodents correlate directly to the peak years of human infections (121).

The transmission activity of SNV differs to some extent from other hantaviruses. SNV is shed and thereby transmitted to other rodents less efficiently than other hantaviruses. Therefore it has been proposed that its transmission among the rodent population requires factors that are not required by other hantaviruses (9).

1.5.2 Viral shedding and antibody responses in rodents

After exposure to hantavirus contaminated aerosols, the viral antigen distributes into many organs and tissues of the rodent. PUUV antigen appears first in lung tissues about 14 days after infection, and a few days after that, the virus is detectable also in liver, spleen, kidneys, salivary glands, urine and faeces (175). According to experimental studies,
the distribution of the antigen is similar regardless of the infection route, i.e. if the vole is infected by i.m. inoculation or normal aerosol route (7). In both cases, the amount of PUUV antigen as well as the viral reproduction and transmission rates are notably decreased with time. The infectious virus persists in the animal’s tissues for at least 15 months, which equates to the maximum lifetime of a bank vole (7, Heikki Henttonen, personal communication). The long-lasting viral persistence in saliva, excreta or tissues has been reported also for IITNV (93), SLOV (77, 159), DCCV (67), SNV (10) and CADV (45). This strongly indicates that the hantavirus infection in rodents is most probably lifelong.

During the persistent and asymptomatic infection, the virus induces an antibody response in the rodents. The PUUV-specific antibody response is directed mainly to N, but also towards G1 and G2 (104, 162). Neutralizing immunity towards hantaviruses is induced by G1 and G2 antibodies. The N protein is highly immunogenic containing several antigenic domains, but the IgG response is directed mainly to its amino-terminal part (104). Hantavirus-specific antibodies appear in the bloodstream about 1 to 3 weeks after the infection, and persist at the same or a slightly lower level for several months (7, 49, 50, 93, 159, 175.), probably for the rest of the rodent’s life.

1.6 Hantavirus infections in humans

Hantaviruses cause two distinct diseases in humans: hemorrhagic fever with renal syndrome (HFRS), and hantavirus pulmonary syndrome (HPS). HFRS is found in Eurasia and HPS causes cases in the Americas. HFRS and HPS resemble each other and capillary leakage syndrome is characteristic of both diseases. A prodromal phase with fever and myalgia as well as hypotension and thrombocytopenia is also common to these two diseases (114).
1.6.1 History of HFRS and HPS

Hantaviruses have most probably existed in the world for millions of years causing human diseases. However, they have been identified only recently after investigating the emerging clusters of diseases with modern virological methods.

The first reference to a disease closely resembling IIFRS dates back to A.D. 960 in a Chinese medicine book (Whang-Jae-Nae-Kyung), which has a disease description suggestive of hemorrhagic fever with renal syndrome (94). In the 20th century the first report of a human viral disease, which is now known as HFRS, is from the Russian clinical records in 1913 (19). After that, HFRS outbreaks were mainly connected to wars. The “Feldnephritis” in British and German troops during World War I was probably caused by hantaviruses (1, 11). Also Japanese military workers invading Manchuria in the 1930s (83), as well as Finnish and German soldiers in Lapland during World War II (65, 158) probably suffered from HFRS. Despite these outbreaks, HFRS did not “hit the headlines” until the 1950s when over 3000 US soldiers serving in the UN troops in Korea were diagnosed with the disease (156). The etiological agent of the HFRS still remained unidentified although many clinical, physiological and pathological investigations were made. Subsequently, the prototype of the whole hantavirus genus, Hantaan virus, was isolated and identified by Dr. HoWang Lee and co-workers in the 1970s in Korea (92).

In 1962 Gajdusek (46) proposed the definition HFRS, which became adopted by WHO in 1983 (171) as a general term for all the hantavirus infections in Eurasia. The term nephropathia epidemica (NE), which refers to the milder form of HFRS found mainly in northern Europe, was introduced in 1945 by the Swedish physician Myhrman. The disease itself was described already in 1934 by two physicians Myhrman and Zetterholm, who worked independently in northern Sweden (122, 123, 124, 178). NE is caused by PUUV (12), but several studies indicate that also SAAV causes mild NF-like HFRS in Eastern and Central Europe (51, 106, 109, 141).

The history of HPS dates back to an outbreak of a new respiratory disease reported in 1993 in the area of Four Corners in the USA where a few Navajo adults died within a short period of time after a brief but fulminant pulmonary illness. The autopsies and the course of the illness of all these cases were similar, but they did not fit to the
pattern of any known disease. Thus no specific diagnosis could be made. The State Department of Health and CDC were notified about these cases and only three weeks later, 24 suspect case-patients were identified. Shortly after that, hantavirus antibodies were detected in the sera of the patients, and the rodent Peromyscus maniculatus was pinpointed as the carrier of the virus (22, 125). A new hantavirus was isolated and called Sin Nombre (Spanish: without name) and the disease was named HPS according to its symptoms (125, 29, 52) (For a review see: 71).

The first viruses, which were associated with HPS in North America, were SNV, BCCV and BAYV (81). In 1995 an outbreak of HPS was reported in Chile and Argentina. The causative agent for these outbreaks was identified and named as ANDV (98). Recently, some evidence for the presence of hantaviruses in the New World before 1990s has been found. The earliest case of a serologically confirmed HPS infection in the Americas has been traced back to 1959 in a 38-year-old male from Utah. Despite suffering from a critical illness with nausea, diarrhea and cough, he survived. At the age of 73, he donated a blood sample, which was tested positive for SNV specific IgG antibodies (43, 81, http://www.cdc.gov).

1.6.2 Transmission to human

Chronically infected carrier rodents are a vector between humans and hantaviruses. The viruses are transmitted to humans most probably via aerosols of virus-contaminated excreta, urine or saliva of the carrier rodents (139). It has been proposed that transmission would also occur via a rodent’s bite (151), and virus-contaminated food or hands. Additionally, individuals who smoke cigarettes are probably exposed more easily to virus infections (116, Olli Vapalahti, personal communication). Due to the infection routes, there is an increased occupational risk of hantavirus infection in farmers, forestry workers, and mammologists (3, 121, 161, 163). Transmission to human is a dead-end for the virus, though the Andes virus has been reported to transmit from human-to-human (131). There are no reports on multiple or consecutive hantavirus infections in humans.
1.6.3 Epidemiology

Hantaviruses are considered as emerging viruses, and they constitute a world wide health problem. Laboratory confirmed cases of HFRS infections have been reported in Albania, Austria, Belgium, Bosnia-Herzegovina, China, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Japan, Korea, Latvia, Netherlands, Norway, Portugal, Russia, Slovakia, Slovenia, Sweden, and Yugoslavia. The most severe cases occur in the Balkans, Korea, China, and Eastern Russia. Currently, HPS infections have been reported in Argentina, Bolivia, Brazil, Canada, Chile, Panama, Paraguay, Uruguay and USA (109, 132, http://www.cdc.gov).

The pathogenic hantaviruses in Europe include PUUV, DOBV and SAAV, and HTNV and SEOV in Asia. Also AMRV-related HFRS has been reported in Far-East Russia. In Eurasia, about 200000 cases of HFRS are reported annually. In China alone 50000 to 100000 cases have been reported annually since 1981 (20, 113, 157), of which thousands are fatal (97). In Korea, Russia and northern and central Europe thousands of cases are reported annually (5, 14, 142). Furthermore, it seems likely that the disease is underdiagnosed in many countries. In Finland the prevalence of PUUV-specific IgG antibodies in humans is approximately 5%, although it may be as high as 20% in some regions. However, on average only about 1000 cases are diagnosed annually (Figure 2), the incidence being 19/100000. When the incidence and prevalence numbers are compared, it can be estimated that only about 13% of PUUV infections in Finland become reported to the authorities (14, 142, 161). Additionally, studies in Estonia (51), Latvia (108), and Sweden (2) reveal that the majority of the PUUV infections are subclinical and remain undiagnosed. In Belgium, the incidence of diagnosed PUUV infections has shown a clear increase in the 1990s continuing to this century (60), which is indicative of the influence of increasing clinical awareness and diagnostics.

Since the first HPS outbreak in 1993 in USA, several HPS infections have been diagnosed all over the Americas, and several hantaviruses have been recognized as causative agents of the disease. By the end of the year 2002, over 1100 HPS cases had been reported in the Americas, with 331 in 31 different states of USA (64, http://www.cdc.gov, http://www.paho.org). SNV causes most of the hantavirus infections
in North America, but infections caused by BAYV, BCCV, MGLV, and NYV have also been reported (http://www.cdc.gov). ANDV infections are the most common cause of HPS in South America. The other human pathogenic hantaviruses in that continent include ARAV, Bermejo, CASV, CHOV, Hu39694, Juquitiba, LANV, LECV and ORNV (91). Despite the intensive hospital care, the mortality of HPS can reach 40%, especially in Sin Nombre infections.

![Graph showing number of cases per year from 1995 to 2002]

**Figure 2.** Laboratory findings of Puumala virus reported in Finland during 1995-2002. Incidences (new cases in a year per 10000 persons) in each year from 1995 to 2002 are: 1.74; 1.76; 1.47; 2.53; 4.46; 1.50; 2.05; 4.95. (Data obtained from http://www.ktl.fi).

### 1.6.4 Pathogenesis

HFRS and HPS are regarded as febrile capillary leak syndromes with a characteristic thrombocytopenia in the early phase of the disease. The major difference between these two syndromes is the primary target organs, the kidneys in HFRS and the lungs in HPS. Hantaviruses replicate predominantly in pulmonary endothelial cells and macrophages, which are not lysed by the viruses (76, 177). The progress of the viremia is not yet totally understood, but according to RT-PCR studies, the duration of the viremia is
very short (62, 134, 140). Nonetheless, hantaviral antigens may be widely distributed to
different organs as demonstrated by immunohistochemical stainings of autopsy
specimens. However the distribution of antigen in the body of patients who have died in
PUUV infection, may be quite limited (58). In HPS patients, hantaviral antigens are found
mainly in the capillary endothelial cells of lungs, kidneys, heart, spleen, pancreas, lymph
nodes, adipose tissue, skeletal muscle, intestine, adrenal gland and brain, but the most
intensive staining is seen in pulmonary endothelial capillaries (177). In IIFRS patients,
most of the viral antigens are detected in kidney, prominently in tubular epithelial cells,
and also in the cortical and medullar areas of the kidneys (54, 66, 82), and in some cases,
antigen has been detected also in the hypophysis (58).

1.6.5 Clinical manifestations of HFRS

The incubation period of HFRS has been suggested to be 2-6 weeks (76, 96, 154).
The severity of symptoms of the disease varies remarkably depending on the causative
agent. HTNV and DOBV cause the most severe form of HFRS, with mortality rates of 5-
12% (5, 94). HTNV infection is also known as Korean hemorrhagic fever (KHF) (94).
Nephropathia epidemicica (NE), which is caused by PUUV and probably by SAAV, is the
mildest form of HFRS. The mortality to PUUV infection is about 0.1% (14, 119), but
according to a Finnish study on NE patients (119) dialysis is needed in 6% of hospitalized
cases. No lethal cases of SAAV infection have been reported (51, 109), but more
information is still needed.

The clinical course of HFRS can classically be divided into 5 distinct phases: febrile,
hypotensive/shock, oliguric, polyuric and convalescent (94, 97). These phases
may overlap or some of them may not appear at all. It has been reported that genetic
factors such as the HLA B8 haplotype, have a major effect on the course of NE (120,
167). IIFRS usually begins with the onset of fever and general symptoms like headache,
myalgia, malaise, weakness and backache (110, 119, 154). Sometimes patients may have
even undergone laparatomy, because the abdominal pain has resembled that seen in acute
appendicitis (48, 172). The hypotensive phase, that lasts a few days, is characterized by
hypotension and hemostatic disturbances such as petechiae, epistaxis, disseminated intravascular coagulopathy (DIC), and shock. Shock is very rare in NE (119, 154, 110), but in KHF, 10-15% of the patients develop shock (94), which may be lethal. In the oliguric phase, the blood pressure usually becomes normalized, but renal insufficiency may develop. Anuria develops in one tenth of the HTNV cases (95), and 50% of the fatalities in KHF occur during this phase (94). Patients who survive for a few days from the oliguric phase and develop a diuretic phase, have usually a favorable prognosis. Daily diuresis of several liters is common and the diuretic phase lasts for 2 to 12 days. Severely ill patients are in danger of succumbing to intravascular volume depletion, electrolyte disturbances or secondary infections. The convalescent phase lasts from weeks to several months, but most of the surviving patients do not experience any long-term problems (For a review see: 96, 97, 111, 114).

In the PUUV infection, the distinct phases of the disease are not so clear, and the disease is generally milder than the disease caused by DOBV or HTNV (119, 168). After three weeks (mean value) of incubation period, nephropathia epidemic starts with a high fever, which is followed by severe headache, nausea, vomiting, abdominal- and back pain and occasionally the patient may develop myopia. Oliguria develops a few days after the first symptoms and lasts about two days. Usual laboratory findings include thrombocytopenia, proteinuria, hematuria, leukocytosis, and increases in serum creatinine, ESR and CRP values. Disturbances in electrolyte balance are rare, but excess liquid intake should be avoided. Hemorrhagic symptoms are extremely rare, however severe renal symptoms may require dialysis in some of the patients. After a short oliguric phase, polyuria develops, and usually at this point the condition of the patient suddenly improves. Depending of the severity of the disease, the convalescent phase may last from 2 weeks to 2-3 months.

1.6.6 Clinical manifestations of HPS

The clinical course of HPS is divided into four discrete phases: febrile; cardiopulmonary/shock/pulmonary edema; diuretic; and convalescent phases (29).
Typically the disease has an incubation period of 1-5 weeks (81, 132), and it begins with fever, myalgia and malaise, including occasionally vomiting. Other symptoms include headache, anorexia, diarrhea and abdominal pain. This febrile phase lasts for less than 2 weeks, typically 3-4 days, and is indistinguishable from the first symptoms of many other viral infections (117). The febrile phase is followed by a sudden onset of pulmonary edema and shock (57). The symptoms in this phase are cough, shortness of breath and dizziness, also hypoxia and hypovolemia are commonly present. Hospitalized patients usually need supplemental oxygen. Shock may be made worse by hypovolemia, which is a result of serum infiltration into lung interstitium and air spaces. Some of the patients die in this phase of shock, hypoxia or circulatory collapse. Patients who survive to the diuretic phase have a much better prognosis. After 2-4 days, rapid diuresis occurs, and at the same time, fever and shock dissipate, and the pulmonary edema disappears. The convalescent phase lasts for several months and is characterized by fever and fatigue. Usually deaths no longer occur during this phase. If the convalescent phase is achieved, long-term recovery is the most probable outcome (For a review see: 38, 52, 87, 114).

1.7 Humoral immune responses to hantavirus infections in humans

In hantavirus infections, the effective immune response has usually already developed at the onset of the symptoms. The immunodominant N protein induces an early and long-lasting humoral immune response, but it does not stimulate a neutralizing antibody response. The protective humoral immune response and the formation of neutralizing antibodies are induced by G2 and G1 (100, 102, 132, 138). As in many other viral infections, virus-specific IgM antibodies are the first evidence of the humoral immune response appearing almost at the same time as the symptoms, and shortly after or almost simultaneously IgG antibodies appear (8, 33, 53, 102, 132). In rare cases IgM antibodies become detectable only on the 6th day after the onset of the first symptoms (73). Early IgG and IgA responses have been demonstrated at least in PUUV, ANDV and SNV infections (70, 101, 132, 162). The IgM antibodies decline to an undetectable level in 2-4 months, whereas IgG antibodies persist for decades (2, 43, 73, 94, 102, 127, 129,
155), probably providing a life-long immunity. So far, no secondary infections in a pre-immune person have been reported.

PUUV-specific IgM antibodies against N, G1 and G2 proteins can be detected in the sera from acute and early convalescent phases. The antibodies are primarily anti-N or anti-G2 antibodies, while anti-G1 antibodies remain at a lower level. The highest titer of IgM antibodies occurs during the acute phase, although in rare cases, low titers are found also in the late convalescent phase (102). However, in the study by Groen et al. (53), anti G1 and N antibodies were found in the acute phase and anti-G2 antibodies in the convalescent and late convalescent phases only. The IgG response is formed firstly towards N, and the titer of anti-N IgG increases more rapidly than the titres of the anti-glycoproteins. However, IgG antibodies to N, G1 and G2 proteins can be detected in most cases already in acute phase samples, although sometimes the titers may be at very low or an undetectable level. The level of IgG antibodies rises to its maximum in the convalescent phase (75, 102, 105, 127).

1.7.1 Serological cross-reactions between different hantaviruses

Cross-reactions of antibodies between the different hantavirus species are common (16, 33, 74, 134, 147), especially between the species that are carried by rodents of the same subfamily. Thus, antibodies against the viruses carried by Murinae (DOBV, HTNV, SEOV), Arvicolinae (PHV, PUUV, TOPV, TULV), and Sigmodontinae (SNV, ANDV) species, cross-react strongly within the group (33, 167). The genetic distances of the different virus species reflect the serological cross-reactivities of the hantavirus nucleocapsid antibodies. For example, DOBV is serologically closely related to HTNV but quite distant to PUUV, and in most cases it can be distinguished from SAAV only by FRNT (18), which is clearly indicated by the sequence homologies of the N proteins of these hantaviruses. Paired differences of the DOBV-N protein to the above mentioned hantaviruses are 39% (PUUV-N), 17.5% (HTNV-N) and 3% (SAAV-N) (144). Antibodies in the sera of acute or early convalescent phases cross-react also in neutralization assays, probably due to cross-reactions of anti-glycoprotein IgM antibodies.
Therefore, sera from late convalescent phase are required for reliable typing of the hantavirus species (102, 105). Studies on serological cross-reactions between hantavirus species have lead to a generalization that only one or two hantavirus antigens are needed for the diagnosis of all hantavirus infections in certain area: SNV antigen in the Americas, and HTNV and PUUV antigens in Eurasia. However, according to Brus Sjölander et al. (16), the HTNV antigen is not sufficient for the diagnostics of DOBV infections, suggesting that three antigens, HTNV, PUUV and DOBV, should be used for diagnosis of HFRS in Eurasia (III).

So far only a few studies concerning the serological cross-reactions with American hantaviruses or between European and American hantaviruses have been published. It seems that PUUV IgG, IgM and IgA anti-N antibodies cross-react more frequently with SNV than with HTNV, SEOV or DOBV. Additionally, the rate of IgG cross-reactivity increases remarkably with time after the onset of the disease (33). In a study by Jenison et al. (70) a high cross-reactivity rate between anti-SNV-N antibodies (the virus was named as Four Corners Virus in the reference) and PUUV-N and PHV-N antigens was reported, whereas no cross-reactions were seen with anti-SNV-G1 antibodies and PUUV-G1 or PHV G1.

1.8 Hantavirus diagnostics

The isolation of hantaviruses from human tissues is quite difficult, and only a few successful cases have been reported (4, 44, 47, 72). Culturing of hantaviruses is laborious and has usually been done either in Vero E6 cells or in rodents (114). This makes virus isolation too laborious for routine diagnostics.

The methods based on detection of viral RNA, such as RT-PCR, are useful in post mortem documentation of hantavirus infections. The virus can be examined by RT-PCR in autopsied tissues, particularly from lungs and kidneys, and this method helps to determine the species of the virus. These methods are not applicable for diagnostics because the viremia in infected patients is very short-lived. According to studies with DOBV (134) and PUUV (139, 140), the viral RNA could be detected only in 40 and 70% of the patients
with acute infection, respectively. In HPS cases, the RT-PCR is more useful, and it can be performed quite reliably in the acute phase serum during the first 10 days of infection (160).

Modern diagnostics of hantavirus infections relies mainly on serological methods, such as EIA (32, 73, 86, 126, 133, 150, 164, 182), IFA (12, 59, 86, 153) or immunoblot assays (150, 181). Recently also rapid immunoblot strip assays (RIBA) (63, http://www.cdc.gov) and immunochromatographic rapid tests (I, III) have been developed. These tests use hantaviral N proteins as the antigen, and the proteins are either native or full-length or truncated recombinant proteins. The native proteins are usually produced by infecting Vero E6 cells with hantaviruses. Recombinant proteins have been expressed in insect cells (17, 118, 164), bacterial cells (32, 41, 55, 133, 182) or mammalian cells (41, 75), after transfection with plasmids or baculoviruses that include the N-coding sequence.

The amino-terminal region of N is the major antigenic site in hantaviral infections (31, 56, 104, 174, 176). Several antigenic epitopes have been found in different parts of the PUUV-N when using epitope mapping (103, 162). Two 15-mer sequences were found as specific binding sites for IgG antibodies in the acute phase of human infection (103). However, there is good evidence that the native or recombinant full-length N protein is the most appropriate antigen for in vitro diagnostic purposes (15, 74). This is probably due to the fact that the anti-N epitope pattern varies remarkably between different individuals (162), and that some of the epitopes may be conformational (103). Thus the full-length N is needed because the truncated proteins do not fold properly to form an epitope.

Some products for in vitro diagnostics of hantavirus infections are commercially available. Commercial EIA methods, that use hantavirus antigens as a mixture or in separated wells are available from Progen Diagnostics (Heidelberg, Germany), and Focus Technologies (Cypress, CA) (formerly MRI, Diagnostics), and commercial IFA methods are available from Progen Diagnostics. A commercial immunoblot assay, which uses hantavirus antigens in different dots, is available from Mikrogen (Martinsried, Germany). These tests detect antibodies to PUUV, HTNV or SEOV (Progen Diagnostics) or to PUUV, HTNV, DOBV or SEOV (Mikrogen) antigens. The EIA test kit from Focus
Technologies uses a cocktail of hantavirus antigens and it claims to be able to detect antibodies to the most clinically relevant pathogenic strains of hantaviruses, i.e. SEOV, HTNV, PUUV, DOBV and SNV.
2 AIMS OF THE STUDY

The aims set for this study were:

1. To produce and purify recombinant hantavirus nucleocapsid proteins to be used as antigens in serological rapid tests, and to evaluate their potential for diagnostic use

2. To develop immunochromatographic rapid tests for the detection of acute hantavirus infections in humans and for the detection of hantavirus specific IgG antibodies in rodents

3. To evaluate the diagnostic potential of the newly developed rapid tests for the detection of human and rodent hantavirus infections
3 MATERIALS AND METHODS

3.1 Antigens

Three recombinant nucleocapsid proteins were used as antigens in the rapid tests (I, II, III, IV, V). PUUV-N was cloned in pACYM1 plasmid and expressed as a recombinant full-length protein in Sf9 insect cells. Recombinant HTNV-N and DOBV-N were expressed as GST-fusion proteins (GST-HTNV-N, GST-DOBV-N) harbouring His-6 tags and thrombin cleavage sites (Figure 3). Cloning of hantavirus N proteins to baculovirus vectors was performed at the Department of Virology, Haartman Institute, University of Helsinki by Antti Vaheri’s research group. PACYM1.2-PUUV-N, a pACYM1 plasmid containing the coding sequence of PUUV Sotkamo strain S segment, was used for transfecting Sf9 cells together with the wild-type baculovirus DNA as described previously (164). For cloning of DOBV-N and HTNV-N, the pAcGHLT-A plasmid (Pharlogen, San Diego, CA) was first modified so that an Ncol site was introduced immediately following the thrombin-cleavage site. The Vent-polymerase-amplified DOBV-N and HTNV-N coding sequences were cloned with standard methods to the modified pAcGHLT-A plasmid using the restriction enzyme sites Ncol (after the thrombin-cleavage site) and KpnI. The cDNA for HTNV-N (strain 76-118) was a generous gift from Dr. Connie Schmaljohn, (USAMRIID, Fort Detrick, MD), and that for DOBV-N was from an isolate from Northern Greece (DOBV/AP) (135). After cleavage with thrombin, an extra Gly-Scr-Ala sequence remained at the amino terminus of the N sequence.

3.1.1 Expression

The recombinant PUUV-N, HTNV-N, and DOBV-N proteins were expressed in Sf9 cells (Spodoptera frugiperda) in Sf900 II medium (Gibco Invitrogen Corporation, Carlsbad, CA) (I, II, III, IV, V). Cells were cultured at +28°C in stirred suspension cultures. When the cells were at logarithmic growth phase the medium was removed by
centrifugation (100-500 x g, 7 min), and the cells were resuspended to ~2 x 10^6 cells/ml with fresh medium. The cells were infected with the baculovirus HTNV-N or DOBV-N clones by adding baculovirus rich Sf900 II medium at 10-15 % (v/v) of total volume. Cells were harvested 72 h post infection by centrifugation (1700 x g, 5 min), washed extensively with PBS (pH 7.4), and resuspended into 2mM EDTA in PBS (pH 7.4). Suspensions (50% v/v) were stored at -70°C with protease inhibitors, which were added as recommended by the manufacturer (Complete™ protease inhibitor cocktail, Roche Diagnostics, Mannheim, Germany).

![Thrombin cleavage site](image)

**Figure 3.** Schematic structure of the GST-HTNV-N and GST-DOBV-N fusion proteins

### 3.1.2 Purification

Isolation and purification of PUUV-N was performed at room temperature from the thawed cell suspension with stepwise urea extraction (I. II. III. IV. V). The protease inhibitors were added to all solutions. The cell suspension was washed three times with 3 M urea in 0.05 M Tris-HCl - 0.05 M NaCl – 1 mM EDTA, pH 8.0 (buffer A), and centrifuged at +4°C (20 000 x g, 15 min). After washings with 3M urea, the cell pellets were suspended into 8.0 M urea in buffer A. After incubating for 1/2 h, the suspension was centrifuged as described above. The supernatant, containing PUUV-N was stored as aliquots at -70°C.
The GST-HTNV-N fusion protein was isolated from the thawed cell suspension by extraction with guanidine hydrochloride (III). Briefly, to remove part of the impurities, the cells were washed once with 4 M urea and twice with 3 M urea in 50 mM phosphate buffer - 0.4 M NaCl (pH 8.0) (Buffer B). The protease inhibitors were added to all solutions. After washings, the cell pellet was resuspended in 6 M guanidine hydrochloride in Buffer B and centrifuged for 20 min with 16000 x g at +4°C. GST-HTNV-N was further purified by Ni-chelate affinity chromatography. The HTNV-N was cleaved from its GST fusion part by thrombin (Amersham Biosciences, Buckinghamshire, England) in the affinity chromatography column. The cleavage was performed at room temperature overnight with 5 mM β-mercaptoethanol – 5 mM imidazole in PBS (pH 7.4). The cleaved HTNV-N was eluted from the column with 8 M urea – 500 mM imidazole – 5 mM β-mercaptoethanol in Buffer B. Finally, the HTNV-N was dialyzed into 20% ethyleneglycol – 20 mM Tris-HCl – 1 mM EDTA (pH 8.0).

The GST-DOBV-N fusion protein was isolated, purified and cleaved as described earlier for the GST-HTNV-N (III). However, there was a difference in the dialysis step. The dialysis was done in two steps to prevent the aggregation of GST-DOBV-N. In the first step, the antigen was dialyzed into Buffer B, and in the second step, the antigen was dialyzed into 20 mM Tris-base – 1 mM EDTA (pH 10.0).

3.1.3 Characterization

A reducing 10% SDS-PAGE was used for assaying the purity and the molecular weight of the PUUV-N, HTNV-N and DOBV-N proteins (I, II, III, IV, V). The protein concentrations were measured with BCA-200 protein assay kit (Pierce, Rockford, IL) according to the manufacturer’s instructions. The biological activity of the purified proteins was confirmed by EIA (164) using verified patient samples.
3.2 Immunochromatographic rapid tests

Design of the rapid tests was based on the lateral flow immunochromatographic test technology. The development of the rapid tests was carried out at the Oy Reagena Ltd. (Siilinjärvi, Finland) adopting the company’s lateral-flow membrane test technology. The tests were designed to detect hantavirus-specific human IgM antibodies or rodent IgG antibodies in serum, plasma or whole blood.

The rapid test device is composed of six individual building blocks, which are the sample pad, conjugate pad, membrane, absorbent pad, adhesive, and plastic holder (Figure 4). Recombinant hantavirus antigens and control reagents were dispensed to the polyester supported nitrocellulose membrane (Whatman International Ltd., Maidstone, UK) using a special liquid dispenser (XYZ Dispenser, BioJet 3000 Quanti / AirJet 3000, Biodot Ltd., Huntingdon, UK). Colloidal gold particles were prepared from aurum tetrachloride with tri-sodium citrate in aqueous solution. This method yields normally approx. 10 nm particles in a homogenous suspension. The particles were coated with either anti-human-IgM (rabbit) antibodies, anti-mouse-IgG (rabbit) antibodies, or PUUV-N antigen under alkaline conditions, and blocked with BSA. The prepared gold-conjugated reagents, which serve as signal transducers in the tests, were absorbed and dried to the conjugated pad made of a glass fibre membrane (Schlisher & Shuell, Dassel, Germany), using the necessary additives. The absorbent pad was inserted in the end of the test device to promote the lateral flow in the test membrane. The components were assembled on the adhesive, cut into 3.9 mm strips and inserted into plastic houlders. Analyses of the samples were done by pipeting first the sample and then running buffer to the sample well. The buffer dissolves the gold-labelled reagents and creates a lateral flow on the nitrocellulose membrane enabling the reactions between antigen and antibodies.

For analysis with the rapid test, 5 μl of the samples (serum, plasma or blood) and two drops (approximately 70-90 μl) of the running buffer were added to the sample well of the test device at room temperature. After 5 minutes, the test results were read visually. According to the test principle, the appearance of two reddish brown lines (test and control lines) in the test window was interpreted as a positive, and appearance of only one line (control line) as a negative result (Figure 4). The test was not valid if the red control
line did not appear in the test window. The non-appearance of the control line indicates that there has been a manufacturing error in that individual test and a new analysis has to be performed.

![Diagram A and B](image)

**Figure 4.** The construct and building blocks of the rapid test device, viewed vertically (A) and horizontally (B).

### 3.2.1 Rapid tests with secondary antibody system

In the rapid tests, which use a secondary antibody system, the test result is visualized by using gold-conjugated rabbit-anti-human-IgM or rabbit-anti-mouse-IgG antibodies. The tests are based on hantavirus-N antigens, and goat-anti-rabbit antibodies serve as the control reagent (Figure 5). Antigen and control reagent are immobilized on the nitrocellulose membrane, and gold-labelled antibodies are dried on the conjugate pad. When the sample and buffer are added into the sample well, IgM or IgG antibodies in the
the blood samples were tested in parallel with three different test batches (III), and 30 samples were tested with only one test batch (I).

3.4.3 Bank vole blood samples

Altogether 204 bank vole blood samples (V) were collected from wild bank voles captured in June (n=78), August (n=21) and October 2002 (n=105) in central Finland. The voles were trapped with Ugglan Special live traps (Grahfab, Hillerstorp, Sweden) in the vicinity of Konnevesi Biological Research Station, University of Jyväskylä, Finland.

These blood samples were collected either with cardiac puncture of anesthetized and subsequently euthanised bank voles (n=21) or from retro-orbital sinus of living bank voles (n=183) with a capillary tube (18 μl, Hirschmann Laborgeräte, Germany). The cardiac puncture samples were stored as 300 μl aliquots at -20°C before analysis with the rapid test or IFA. The capillary tube samples were treated in two different ways depending on the sample panel. Of these samples, 105 were assayed immediately after sampling with the rapid test in a field laboratory, and few days later with IFA. The rest of the samples (n=78) were collected from bank voles tested previously as negative for PUUV specific IgG antibodies with IFA. The animals, which were tested as PUUV-IgG positive with IFA, were caged in the same room with the negative animals for two days before the positive ones were separated. This enabled the horizontal transmission of the PUUV among the bank voles. Three weeks after the first IFA test, the samples were collected again from the 78 previously PUUV-IgG negative bank voles, and stored for two days in the capillary tubes at +4°C. Then the blood was removed from the capillaries and frozen as 5 μl aliquots at -20°C for later use by the rapid test and IFA. The donor voles were housed in single vole cages in a laboratory for the entire time between the first and second sampling.

The samples collected with the capillary tubes partially clotted on the walls of the tube. Therefore, after collection, the samples transferred into 2 ml cryogenic vials (Corning, Cambridge, MA). Although the samples were removed from the capillary tube
into the vial, they still tented to form clots. In these cases, the clot-free phase was used for analysis.

3.4.4 Serum samples from bank voles and other animals

Altogether 21 bank vole (Clethrionomys glareolus), 5 rabbit, 5 sibling vole (Microtus rossiaemeridionalis), and 26 lemming (Lemmus lemmus) serum samples were used. The bank voles were caught in autumn 1991 in Luhanka, central Finland, and 7 of them were naturally infected with PUUV. One rabbit was infected by inhalation with Prospect Hill virus, the other with Khabarovsk virus, and the third with Tula virus at SMI (Stockholm, Sweden) (165). Two rabbits had been immunized with recombinant GST-fusion protein including N-terminal PUUV nucleocapsid protein (N) (162). The sibling voles were experimentally infected with Tula virus at the Department of Virology, Haartman Institute, University of Helsinki, Finland (165). Of the lemming samples, 20 were from wild Norway lemmings, and 6 were from lemmings infected experimentally with Topografov virus at the Ojajoki Field Station, Finnish Forest Research Institute, Finland (166). The lemmings were caught in the Kilpisjärvi region, Finnish Lapland.

3.5 Reference methods

3.5.1 PUUV IgM EIA

The PUUV IgM μ-capture EIAs based on recombinant PUUV-N antigen was used as a reference method (15, 73, 164). The peroxidase-labelled monoclonal antibody (MAb) 1C12 (99) was used to detect the antigen (Figure 7), and the serum samples were diluted 1:200. EIAs were done at the Department of Virology, HUCH Laboratory Diagnostics, Helsinki, Finland; and at the Swedish Institute for Infectious Disease Control, Stockholm, Sweden.
3.5.2 *PUUV* IgG IFA

The IFA was used to detect PUUV-specific IgG antibodies in human and bank vole samples (13, 59). Briefly, a mixture of PUUV-infected and uninfected Vero E6 cells was acetone fixed on slide spots, and stored at -70°C until analyzed. The serum samples were diluted 1:20 and incubated on slides. The results were detected using fluorescein isothiocyanate conjugated to anti-human IgG or to anti-mouse-IgG antibodies. The analyses were performed at the Department of Virology, HUCH Laboratory Diagnostics / Haartman Institute, University of Helsinki, Helsinki, Finland.

3.5.3 Confirmation of DOBV and HTNV samples

The DOBV-specific IgM-positive serum samples were from HFRS patients diagnosed in Bosnia-Herzegovina, Slovenia and Greece. All these samples were confirmed as DOBV-specific with FRNT (5, 18, 105) at the Swedish Institute for Infectious Disease Control. The HTNV-specific IgM-positive serum samples were collected from Korean patients suffering from acute HTNV infection and diagnosed serologically in the WHO collaborating center of HFRS, Seoul, Korea.

![Diagram](image)

Peroxidase-conjugated MAb 1C12

PUUV-N antigen

PUUV-N–specific IgM antibody from patient serum

Goat anti human IgM antibody

Solid support

**Figure 7.** Basic principle of the PUUV IgM μ-capture EIA.
4 RESULTS

4.1 Hantavirus N antigens

4.1.1 Expression

Three hantavirus N proteins were expressed in Sf9 insect cells (*Spodoptera frugiperda*) (164). PUUV-N was expressed successfully as a full-length recombinant protein (I, II, III, IV, V), whereas DOBV-N and HTNV-N were expressed as a chimeric protein with a GST fusion partner (III). The cell culture yields of DOBV-N without the GST partner were very low, and therefore the GST partner was inserted to both IITNV and DOBV constructs to enhance the protein expression. The expressions of GST-HTNV-N and GST-DOBV-N in cell cultures enhanced but remained still at a lower level than that of native PUUV-N. Cell culture with 200 ml volume yielded approximately 2-10 mg PUUV-N protein.

4.1.2 Purification

PUUV-N protein was successfully isolated from the Sf9 insect cell lysate by a stepwise urea extraction (I, II, III, IV, V), and GST-DOBV-N and GST-HTNV-N were extracted from cells with guanidine hydrochloride (III). Part of the impurities were removed from the lysates with 4 M urea, and the recombinant proteins were extracted from cells without any significant loss of the N proteins. The final purification of the hantaviral N proteins was achieved using affinity chromatography. To attain 100% cleavage of the GST partner from the N-proteins, thrombin had to be used in excess amounts.

After the imidazole elution, three major bands were seen in SDS-PAGE (Figure 8, lane 5). Probably due to very strong binding of the cleaved N protein to the affinity chromatography column, GST and N proteins detached partially at the same time from the
column. The two additional bands appeared only after thrombin treatment (Figure 8, lane 2 vs. lane 5), and they were most likely GST and its cleavage residue. Affinity chromatography removed most of the other impurities from the solution. The antigenic activity of the N proteins was remarkably reduced when the fusion partner was not cleaved from the N proteins. This phenomenon was clearly seen as greatly lowered sensitivity of the rapid tests (data not shown).

4.1.3 Characterization

The protein expression in Sf9 cells, extraction of the proteins from the Sf9 cells, the purification (I, II, III, IV, V), and the thrombin cleavage (III) were monitored with SDS-PAGE (Figure 8). The theoretical molecular weights of the hantavirus N proteins are 49.5 kD for PUUV-N, 48.1 kD for HTNV-N, and 48.2 kD for DOBV-N (http://srs.cbi.ac.uk), and according to SDS-PAGE the molecular weight of the expressed proteins were <50 kD. In Figure 8, GST-DOBV-N is presented as an example. GST-HTNV-N and PUUV-N (Figure in I) showed similar patterns in the SDS-PAGE gels as GST-DOBV-N. When the desired purification rate was accomplished with extraction and affinity chromatography, the protein concentration of the solution was measured. If the concentration was too dilute, the solution was concentrated through a centrifugal filter (Ultrafree®-4, Millipore, Millipore Corporation, Bedford, MA), and the protein concentration was measured again. All three N proteins were found to be largely insoluble in physiological conditions, as described earlier for PUUV-N (164).

With EIA and verified serum samples, the diagnostic potential of the purified proteins was determined to be adequate (data not shown).
4.2 Rapid test development

The development of the serological rapid tests was done at the Oy Reagena Ltd. adopting the company’s lateral-flow membrane test technology. The antigen concentration, the additives and blocking agents, the preparation of gold-labelled conjugates, the material and pore size of the membrane, the composition of the running buffer, and the overall assaying conditions were optimized for different rapid tests. (These cannot be discussed in detail, because they are trade secrets.) As a result of this development work, rapid tests for detection of human (I, II, III, IV) and rodent (V) hantavirus infections were created (Figure 9).
the reference method as PUUV-specific IgG positive but IgM negative, and all of these four were negative with the rapid test. One unfrozen sample gave a borderline result with IgM EIA and a positive result with the rapid test. The second sample from the same patient collected and tested one month afterward was clearly PUUV IgM and IgG negative with reference methods, and it was categorized as negative with EIA in the statistical calculations.

Table 3. POC PUUMALA® rapid test results with fresh human serum samples (n=103) compared to the results of the IgM μ-capture-EIA reference method (I, IV). The borderline result with EIA is considered as negative.

<table>
<thead>
<tr>
<th>PUUV-IgM μ-capture-EIA</th>
<th>POC PUUMALA®</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
</tr>
<tr>
<td>Positive</td>
<td>27</td>
</tr>
<tr>
<td>Negative</td>
<td>149</td>
</tr>
</tbody>
</table>

The positive predictive value for the PUUV rapid test varied between 97% and 100% with frozen serum samples (if the IgM EIA borderline result was considered as negative) and was 92% with unfrozen samples. The negative predictive value for the PUUV rapid test was 100% with unfrozen serum samples, and varied between 97% and 99% with frozen serum samples (I, III, IV).

4.3.2 Analytical performance of the PUUV rapid test with fingertip blood samples

Altogether 130 fingertip blood samples were collected from human volunteers (I, III). In addition to fingertip blood, also serum samples were taken from 100 volunteers,
and all of these serum samples were determined as PUUV-specific IgM negative with the reference method (III). However, six of them were PUUV-specific IgG positive with IFA. The 30 blood samples, which were tested only with one test batch, were not confirmed as negative with the reference methods, but the volunteers did not have any symptoms of NE according to their interview answers (I).

The fingertip blood samples showed 99% to 100% specificity with the rapid test. Only two samples gave a false-positive result with one test batch, and these two samples were PUUV-specific IgG negative according to IFA. Since none of the volunteers was positive for PUUV-specific IgM antibodies when tested with the reference method, fingertip blood was spiked (1:2) with PUUV-specific IgM-positive sera (n=40). All these spiked samples gave a positive result with the rapid test.

4.3.3 Comparison of the PUUV rapid test with commercially available EIA tests

The analytical performance of the POC PUUMALA® rapid test was compared to two commercially available EIA tests: the Hantavirus (Puumala) IgM ELISA (Progen, Heidelberg, Germany) (II) and the Hantavirus ELISA IgM (MRD Diagnostics, Cypress, CA; the company’s current name is Focus Technologies) (IV). The samples used in the evaluation were either frozen (n = 438) or were analyzed in the diagnostic laboratories at routine intervals (n = 121). The comparisons were carried out in two different sites (Kuopio and Stockholm), therefore different samples were used when comparing the rapid test to two different commercial EIA tests.

Comparison of the analytical performances of the POC PUUMALA® rapid test and the Progen Hantavirus (Puumala) IgM ELISA was done with 235 old frozen serum samples and 48 fresh serum samples, which were analysed in routine diagnostic intervals in parallel with the rapid test and Progen IgM ELISA (II). The samples that gave the same result with Progen EIA and all the rapid test readers were not tested with reference method. Only the discrepant samples, samples that were not agreed by all rapid test readers, or samples that gave grey-zone result with EIA were tested with the reference method. Five individual readers interpreted the rapid test results of the frozen sample,
while the fresh samples were interpreted by only one trained technician. When tested with frozen samples the concordance of the results of POC PUUMALA® rapid test and Progen Hantavirus (Puumala) IgM ELISA varied from 93% to 96%. The sensitivity of the rapid test varied between 98% and 100% among independent readers, while it was 94% with Progen ELISA. The specificities for the rapid test varied between 95% to 100%, while being 97% for the Progen ELISA. The mean efficacy between the five individual rapid test readers was 98.6% and for Progen ELISA it was 96% (Table 4). There were 14 samples which gave a grey-zone result with the Progen ELISA, and these samples were not included in Table 4 or in the statistical calculations described above, because of the uncertain interpretation of the result.

When tested with fresh samples the concordance of the results with the two tests was 94%, while three samples gave discrepant results. Two of these three samples gave matching results with Progen ELISA and the reference method, and one sample gave a matching result between reference method and rapid test.

**Table 4.** Comparison of the POC PUUMALA® rapid test and Progen IgM ELISA. The number of frozen and thawed PUTIV-specific IgM-positive samples was 170 and IgM-negative negative samples was 101 (II). Grey-zone results with Progen EIA (n=14) are not included in the table (see paragraph 4.3.3.).

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Efficacy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rapid test reader 1</td>
<td>100 %</td>
<td>95 %</td>
<td>98 %</td>
</tr>
<tr>
<td>Rapid test reader 2</td>
<td>98 %</td>
<td>100 %</td>
<td>99 %</td>
</tr>
<tr>
<td>Rapid test reader 3</td>
<td>98 %</td>
<td>99 %</td>
<td>99 %</td>
</tr>
<tr>
<td>Rapid test reader 4</td>
<td>98 %</td>
<td>100 %</td>
<td>99 %</td>
</tr>
<tr>
<td>Rapid test reader 5</td>
<td>99 %</td>
<td>96 %</td>
<td>98 %</td>
</tr>
<tr>
<td>Progen IgM ELISA</td>
<td>94 %</td>
<td>97 %</td>
<td>96 %</td>
</tr>
</tbody>
</table>
Comparison of the MRI. ELISA and the POC PUUMALA® rapid test was done with 131 PUUV-specific IgM positive and 145 negative serum samples (IV). The negative samples were collected from patients with suspected but not diagnosed NE infection or patients with other acute viral infections. The μ-capture EIA was used as the reference method. The specificity of the rapid test was 97.9% and the sensitivity 99.2%, whereas they were 94.3% and 95.7% for MRL EIA, respectively. The rapid test showed slightly higher efficacy than the MRL EIA (Table 5). The positive predictive value for the rapid test was 97.7% and 95.7% for MRL EIA. The negative predictive value for the PUUV rapid test was 99.3% and 94.3% for MRL EIA.

Table 5. Comparison of the results of POC PUUMALA® rapid test and MRL ELISA test to the IgM μ-capture EIA reference method (IV). Of the samples, 58 PUUV-specific IgM negative and 15 positive were assayed only with POC PUUMALA® and the reference test:

<table>
<thead>
<tr>
<th></th>
<th>Positive</th>
<th>Sensitivity</th>
<th>Negative*</th>
<th>Specificity</th>
<th>Efficacy</th>
</tr>
</thead>
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<tr>
<td>μ-capture-EIA</td>
<td>131</td>
<td></td>
<td>145</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MRL Hantavirus ELISA IgM</td>
<td>111 (of 116)</td>
<td>95.7%</td>
<td>82 (of 87)</td>
<td>94.3%</td>
<td>95.0%</td>
</tr>
<tr>
<td>POC PUUMALA®</td>
<td>130 (of 131)</td>
<td>99.2%</td>
<td>142 (of 145)</td>
<td>97.9%</td>
<td>98.6%</td>
</tr>
</tbody>
</table>

*Including borderline results

4.3.4 Interference of rheumatoid factor or IgM antibodies specific to other viral infections with PUUV rapid test and commercially available EIA tests

The interference of IgM antibodies specific to other viral infections or rheumatoid factor (RF; anti-IgG-Fc IgM) with PUUV rapid test and commercially available EIA tests was evaluated with 77 serum samples (I, II, IV). Samples were collected from patients
4 Results

suffering from acute Epstein-Barr, human parvovirus, rubella virus, dengue virus, Sindbis virus, measles virus, varicella-zoster virus, influenza A or cytomegalovirus infection or patients positive for RF. Of these 77 samples, 76 showed a negative result with the rapid test, and only one RF-positive sample showed a positive result.

There were 40 samples containing unspecific anti-viral IgM antibodies, which were assayed with MRL EIA and with the rapid test. With these samples, the rapid test detected all as negative, whereas the MRL EIA found two of them as positive and three as borderline (IV). Additionally, there were 10 samples, which were assayed with Progen IgM ELISA and the rapid test. The rapid test detected also all of these samples as negative, but Progen IgM ELISA found only 8 negatives, and gave two grey-zone results (II).

4.4 Evaluation of the analytical performance of the DOBV- and HTNV-specific and hantavirus combination rapid tests

The analytical performances of the POC DOBRAVA®, POC HANTAAN® and POC HANTA® rapid tests were evaluated with 105 serum and 100 fingertip blood samples (III). These tests were developed for the detection of acute Dobrava or Hantaan virus infections or to detect all Eurasian hantavirus infections in humans, respectively. The serum samples were confirmed with reference methods as positive for PUUV IgM (n = 27), DOBV IgM (n = 25), and HTNV IgM (n = 25), and as negative for hantavirus IgM (n = 28).

4.4.1 Analytical performance with serum samples

Altogether 77 serum samples from patients with acute-phase PUUV, DOBV or HTNV infections, and 28 serum samples from patients not suffering from HFRS and confirmed as hantavirus IgM negative, were tested in order to evaluate the analytical performances of the rapid tests (III). The positive samples had been frozen for several years prior to testing.
Helena Sirola: Serological rapid tests for detection of human and rodent hantavirus infections

The intra and inter assay variations of the rapid tests were evaluated by testing the same positive and negative samples several times. The samples were tested in parallel with several rapid tests, and no batch variation was observed.

Analysis of the samples gave sensitivities of 96% for POC DOBRAVA®, 100% for POC HANTAAN®, and 93%, 86% and 80% for POC HANTA® with PUUV, DOBV and HTNV samples, respectively. The specificities were 100% for POC DOBRAVA®, 96% for POC HANTAAN®, and 96% POC HANTA® as judged from the panel of non-HFRS samples.

The positive predictive values of POC DOBRAVA®, POC HANTAAN®, and POC HANTA® rapid tests were 100%, 96%, and 99%, respectively. Negative predictive values were 97% for POC DOBRAVA®, 100% for POC HANTAAN®, and 71% for POC HANTA®.

4.4.2 Analytical performance with fingertip blood samples

Using 100 fresh fingertip-blood samples, the specificities of the different hantavirus rapid tests were 98-100% for POC DOBRAVA®, 97-100% for POC HANTAAN®, and 98-100% for POC HANTA® (III). Due to the non-appearance of the control line, 1% of DOBV rapid tests and less than 1% of HTNV tests were rejected. Of the samples, 1% gave false-positive results with HTNV rapid tests and 0.7% with the combination test and none with DOBV rapid tests. The rejected tests were not repeated, and each serum sample was tested three times with every rapid test.

Since none of the volunteers reported having had hantavirus infection, fingertip blood samples were spiked (1:2) with DOBV-specific (n = 10), and HTNV-specific IgM-positive sera (n = 4). All the spiked samples showed positive results with the corresponding rapid tests, and no batch variation was observed.
4.5 Cross-reactions between PUUV-, DOBV-, and HTNV-specific IgM positive serum samples and PUUV-, DOBV-, HTNV-, and hantavirus combination rapid tests

The cross-reactions between different hantavirus rapid tests and different hantavirus IgM-positive serum samples were studied with 105 human serum samples (III). Of the samples, 27 were positive for PUUV, 25 for DOBV and 25 for HTNV IgM antibodies, and 28 were negative for hantaviral antibodies according to reference methods.

Analysis of the cross-reactions showed that POC PUUMALA® was able to detect only 5 of the 25 DOBV-positive samples and 3 of the 25 HTNV-positive samples. POC DOBRAVA® did not detect any of the 27 PUUV-positive samples, but detected 19 of the 25 IITNV-positive samples. POC IIANTAAN® showed a positive result with 9 of the 27 PUUV-positive samples and with 24 of the 25 DOBV-positive samples. POC HANTA® detected 25, 21 and 20 of the 27 PUUV-, 25 DOBV-, and 25 HTNV-positive samples, respectively. From the 28 negative samples, POC PUUMALA® and POC DOBRAVA® detected no false positives, whereas POC IIANTAAN® and POC HANTA® each detected one false-positive sample (Table 6).

Table 6. Percentages of the positive results with PUUV-, DOBV-, HTNV-, and combination rapid tests when assayed with confirmed PUUV-, DOBV-, and HTNV-specific IgM positive and hantavirus IgM negative serum samples (III). The IgM μ-capture-EIA and FRNT were used as the reference methods.

<table>
<thead>
<tr>
<th>Serum samples</th>
<th>Rapid tests</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PUUV rapid test</td>
</tr>
<tr>
<td>PUUV (n=27)</td>
<td>96%</td>
</tr>
<tr>
<td>DOBV (n=25)</td>
<td>20%</td>
</tr>
<tr>
<td>HTNV (n=25)</td>
<td>12%</td>
</tr>
<tr>
<td>Negative (n=28)</td>
<td>0%</td>
</tr>
</tbody>
</table>
4.6 Evaluation of the rapid tests for detection of hantavirus IgG antibodies in rodents

Altogether 204 bank vole blood samples, divided into three different panels, were used to evaluate the analytical performance of rapid test for detection of PUUV-specific IgG antibodies in bank voles (V). Of the 204 bank vole blood samples, 54 were determined to be PUUV-specific IgG positive and 150 as negative with the IFA (13, 59) reference method.

Of the blood samples, 105 were assayed freshly (=immediately after sampling). Of these samples, 16 were positive for PUUV specific IgG antibodies and 89 were negative according to IFA. All of the rapid test analyses were done in the field laboratory at room temperature. The rapid test results were interpreted by 2 individual readers, who agreed with each other about the rapid test interpretations and with the IFA results, for every blood sample tested.

Furthermore, 21 samples were analyzed with the rapid test using undiluted frozen and thawed bank vole blood, which had been stored as 300 μl aliquots. The analysis was done in the laboratory setting. Of these samples, 7 were PUUV-specific IgG positive and 14 were IgG negative with the IFA reference method. The rapid test results were interpreted by 5 individual readers, all of whom were in total concordance with the reference method and with each other.

For the analysis with the rapid test, 78 blood samples were stored in capillary tubes at +4°C for 2 days and then frozen in 5 μl aliquots. After thawing, the samples were diluted 1:10 with PBS and assayed with the rapid test. Of these samples, 31 were positive for PUUV-specific IgG antibodies and 47 were negative according to IFA. The rapid test results were interpreted by 6 individual readers, and the assay performance parameters were calculated individually for every reader. Values for the specificity varied from 92% to 100%, and values for the sensitivity from 81% to 90% between individual readers (Table 7). Of the 78 samples, 13 (17%) had discrepant interpretations among the 6 readers. The positive predictive value varied from 87.5% to 100% between individual readers, with the mean value being 93.4%. The negative predictive value varied from 88.7% to 93.8% between individual readers, the average value being 91.6%.
4 Results

Table 7. Results of the rodent hantavirus IgG rapid test tested with 78 bank vole blood samples (V). The results were interpreted by six persons. According to the PUUV-IgG-IFA reference method, there were 31 PUUV-specific IgG antibody positive and 47 negative bank vole blood samples.

<table>
<thead>
<tr>
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<th>Mean (%)</th>
<th>Range (%)</th>
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<tbody>
<tr>
<td>Positive predictive value</td>
<td>93</td>
<td>88-100</td>
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<tr>
<td>Negative predictive value</td>
<td>92</td>
<td>89-94</td>
</tr>
<tr>
<td>Specificity</td>
<td>96</td>
<td>92-100</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>87</td>
<td>81-90</td>
</tr>
<tr>
<td>Efficacy</td>
<td>91</td>
<td>90-93</td>
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Additionally, 26 Norway lemming and 5 sibling vole serum samples were assayed with the rapid test for detection of hantavirus antibodies in rodents (V). According to the PUUV-IgG-IFA reference method, 3 of the 6 TOPV-infected lemming samples and 1 of the 5 TULV-infected vole samples were positive. The IFA and rapid test were assayed on the same day from thawed serum samples. Both of the rapid test readers interpreted all of the four IFA-positive samples as positive. Of the 27 IFA-negative samples, one reader interpreted all as negative, whereas the other reader interpreted one sample as positive and 26 samples as negative. The false-positive sample was collected from sibling vole.

4.7 Evaluation of the rapid test with a universal detection system

The analytical performance of the rapid test with a universal detection system was evaluated using 56 serum samples from humans and different animal species (Data not previously published). According to IFA reference method, there were 25 human, 5 rabbit, 4 lemming and 7 bank vole serum samples positive for PUUV-specific IgG antibodies. Additionally, there were 1 human and 14 bank vole sera, which were negative for PUUV-
specific IgG antibodies. Lemmings were infected with TOPV and rabbits were immunized with PUUV, whereas humans and bank voles were naturally infected with the virus.

The rapid test detected all the positive rabbit and lemming serum samples. The rapid test result was positive with 22, and negative with 4 human serum samples. Of these, 3 were false-negative results, but none of them was false positive. With the bank vole serum samples, the rapid test detected 5 as positive and 16 as negative, of which 2 were false-negatives and none was false positive (Table 8).

<table>
<thead>
<tr>
<th>Table 8. Results of rapid test with universal detection system compared to IFA reference methods.</th>
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<tr>
<td></td>
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<tr>
<td>Human sera</td>
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<td>Rabbit sera</td>
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<td>Lemming sera</td>
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<td>Bank vole sera</td>
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5 DISCUSSION

5.1 Epidemiological and diagnostic aspects of acute hantavirus infections

Hantaan, Dobrava, and Puumala viruses are the main causative agents of HFRS, including its milder form NE, in Eurasia. The severity of the disease varies remarkably from an asymptomatic infection to fulminant hemorrhagic shock and death, depending on which virus has caused the infection. Early and correct diagnosis is an important factor for successful treatment and management of the disease. In the hantavirus infections, the CRP is high, which is usually indicative of a bacterial infection. With the correct diagnosis the unnecessary or even inappropriate medication such as excess liquid intake, unnecessary antibiotic treatment, or laparotomy can be avoided. The NE is relatively easy to diagnose, because the rise of PUUV-specific IgM antibodies is a clear marker of the acute infection, and these antibodies appear usually almost at the same time as the symptoms. Serology does not always provide such a clear marker of acute virus infections. For example in *Borreliia burgdorferi* infection (Lyme borreliosis) the specific antibodies may appear late or not at all and antibodies from an earlier infection may confuse the diagnosis. In Sindbis virus infection (Pogosta disease), the IgM antibodies are present in the first sample only in every third patient (89).

To reduce the HFRS cases and to minimize the risk of succumbing to the infection, the notification of the population at risk as well as the epidemiological studies of hantavirus infections among carrier rodents are important prophylactic measures. At present, the mortality in PUUV infection is around 0.1% (14) though in HTNV or DOBV infections it is around 3-12% (5, 94), and a great number of patients require hospitalization, even intensive care. In the year 2002, the annual incidence of NE cases in Finland was higher than ever before (2556 / 10 000) and there are reports of a sudden increase of PUUV infections in Belgium (60). However, studies of PUUV prevalence and incidence in Finland (14), Estonia (51), Latvia (108), and Sweden (2) indicate that in all these countries the majority of the PUUV infections are subclinical or undiagnosed. Most likely also numerous other hantavirus infections are not diagnosed annually worldwide...
and there probably are many nations, which are not aware that hantaviruses exist and circulate also in their country. Thus more efficient diagnostic methods and increasing knowledge about hantavirus infections are still needed worldwide. The rapid tests for the detection of human and rodent hantavirus infections, which were developed in this study, offer one answer to the need for more efficient, easy and rapid diagnostics for individuals who suffer from hantavirus infections, and people who nurse or study infections in humans or rodents, respectively.

5.2 Hantavirus nucleocapsid proteins as antigens in diagnostic tests

To date there are only a few commercially available assays suitable for the detection of hantaviral antibodies in human samples. These tests utilize EIA-, IFA-, or immunoblot formats, which require a diagnostic laboratory environment. The commercial EIA tests use either single or a cocktail of recombinant hantavirus N proteins as antigens, and they are based on an indirect ELISA method. Due to these methodological limitations, commercial EIAs are probably more susceptible to interference of RF and other classes of specific antibodies than the μ-capture-EIA, which was used as a reference method in the thesis. It has earlier been reported that native or recombinant full-length N proteins provide better analytical characteristics in serological tests, than if truncated forms of the protein are used as the antigen (15, 74). In this thesis, new serological rapid tests for the diagnosis of acute PUUV, DOBV or HTNV infection in humans were introduced, and all these rapid tests use baculovirus-expressed, full-length hantaviral nucleocapsid proteins as the antigens.

The production of recombinant proteins using baculovirus expression system has been shown to be simple, safe and an inexpensive method for preparing milligram quantities of the PUUV-N (164), HTNV-N and DOBV-N proteins. In our study, the expression levels of all three antigens were high, and the proteins were largely insoluble in physiological conditions, as described earlier for PUUV-N (164). Thus, 8M urea was used as the eluting solution when proteins were eluted from the Ni-column. The GST fusion protein and N proteins showed similar affinity to Ni-chelate and therefore GST
could not be efficiently removed from the protein solution with other impurities. Fortunately, we could not see differences in the specificity of the rapid test when using DOBV-N and HTNV-N as antigen with or without the GST residue present as an impurity in the antigen solution. However, the sensitivity of the rapid test was decreased when the GST residue was not cleaved from the N protein. In this case, the GST may mask the antibody binding or inhibit the correct folding of the conformational epitopes of the antigen.

5.3 Hantavirus rapid tests for humans

The performance of POC PUUMALA® was compared to the performance of the commercial EIA tests (Progen IgM ELISA and MRL ELISA). The rapid test showed equal or slightly better analytical performance than the commercial tests. The higher sensitivity of POC PUUMALA® may, at least partially, be explained by the use of various hantavirus antigens in the MRL ELISA (cocktail of hantavirus N proteins), because the use of homologous antigens has earlier been proven to be important for optimal serology (16, 73). POC DOBRAVA® and POC HANTAAN® showed similar analytical performance in the detection of DOBV or HTNV specific IgM antibodies than POC PUUMALA® with PUUV IgM specific samples. However, the sensitivity and specificity of POC HANTA®, which uses the mixture of PUUV-N, DOBV-N, and HTNV-N as antigens, was lower than the corresponding tests with single antigen. This also supports the proposal, that the antigens used in serology should be homologous. Another reason for the lower ability of the combination test to detect the positive samples is probably due to the tendency of the N proteins to aggregate. Hantaviral N proteins easily form trimers and multimers (80), and when the proteins are mixed they may aggregate with any of the other proteins involved. This may lead to blocking of certain epitopes and emphasizing of others. The POC HANTA® rapid test was developed to detect different hantavirus infections in patient with one test. The test uses a mixture of three hantavirus antigens and relies on serological cross-reactions. With improved sensitivity it would serve as a valuable diagnostic tool in areas where multiple hantaviruses circulate.
The rapid test results are based on the subjective interpretation of the reader and individual differences occur due to the human errors. In some cases the formation of a “ghost line”, which appears as a very faint and hard-to-interpret test line, may interfere with the interpretation (79). The line may be formed as a consequence of repeated freezing and thawing of the serum samples, causing the clotting of the unspecific antibodies. Also other serum proteins may bind to the antigen, leading to the partial blocking of liquid flow and the emergence of the “ghost line”. The ghost line is a problem especially for inexperienced readers. It was difficult to provide written instructions to teach how to distinguish the ghost line from real positive reaction. Therefore, an additional cut-off control, which is a test device including the line with the faintest possible positive reaction, proved to be a good control for correct interpretation, although this method has also been criticized (90).

It is sometimes impossible to distinguish the difference between a faint positive reaction and a “ghost line” in the rapid tests. The avidity of the antibodies towards the antigen is a critical feature in this test format. Assaying the test with sample, which contains antibodies with very low avidity towards the antigen, usually leads to a faint or negative reaction. The avidity of IgM antibodies may be very low due to individual genomic differences, and the fact that affinity maturation has not yet occurred (23, 59, 152).

5.4 Hantavirus rapid tests for rodents

Throughout Europe there is a major annual variation of hantavirus prevalence among rodent populations, depending on geographical region, food supply, predators, weather, etc. (7, 13, 27, 39, 128). Since the human cases of hantavirus infections correlate directly with the rodent densities or the rate of hantavirus prevalence in rodent populations in certain area (14, 121, 136), the screening of rodent population for hantavirus antibodies may help in anticipating or controlling the human hantavirus outbreaks. At present EIA and IFA arc among the most widely used screening methods for rodent samples (13, 59, 61, 78). Unfortunately, the present methods are poorly adapted to
field-conditions due to the need for sophisticated laboratory equipment, trained personnel and their long testing time.

To meet the demands of field-diagnostics, the rapid test for detection of hantavirus-specific IgG antibodies from rodents was developed. It is fast and easy to use, it works without sophisticated laboratory equipment outside the ordinary diagnostic laboratory, and it can be assayed with whole blood samples by quickly trained people, who are not used to work with laboratory instruments. Additionally, it was proved that the test can be used to detect antibodies from a variety of rodent species. The anti-mouse-antibodies used in the test showed adequate cross-reactivity to samples collected from rodents from the Arvicoline clade (voles, and lemmings), and they may be able to cross-react with antibodies from several other rodent species.

The rapid test showed a very good analytical performance when blood samples were studied, and the best results were achieved when fresh blood samples were tested. If blood samples were frozen or stored at +4°C in capillary tubes for a few days the test showed poorer performance, which might be due to the precipitation of antibodies or vaporization or lyophilization of the samples. Interpretation of the rodent IgG rapid test was easier than that of the human IgM test, because the “ghost line” did not present a problem with this test. This is probably partly due to the fact that IgG antibodies have undergone affinity maturation and show higher specificity and avidity and less heterogeneity towards their specific antigens than the earlier IgM antibodies (23). The rodent IgG rapid test provides a convenient and efficient screening method for bank vole populations, and it may act as a tool for predicting the coming outbreaks of NE, or even controlling the PUUV transmission to humans. Additionally the rodent IgG test may be used as a tool to find new still unknown hantaviruses circulating in the rodent populations. There are a few hundred rodent species known in the world, and one cannot say certainly if still unknown hantaviruses are circulating among the rodent populations. The rapid test showed some cross-reactivity, at least with TOPV-specific and TULV-specific antibodies, and it is not clear how many other hantavirus antibodies it is able to detect.

The test using a universal detection system does not demand samples from specific animal species. Therefore if used with varying antigens it would prove to be a convenient tool in screening different animal populations and searching for new virus host
species. However, the system places high demands on the quality of the antigen. For this reason the preparation of these tests proved to be very expensive, and the system would not be frequently used or commercially available until more inexpensive methods to prepare gold-conjugated antigens can be developed.

5.5 Serological cross-reactions between hantaviruses

The rapid test for detection of hantavirus-specific IgG antibodies from rodents uses PUUV-N as the antigen. To assess the properties of the rodent rapid test to detect antibodies to related hantaviruses in other rodent species, serum samples from Norway lemmings and sibling voles were analyzed with the test. The lemmings and voles were experimentally infected with TOPV and TULV, respectively. TOPV nucleocapsid protein (TOPV-N) shows a 13% difference in amino acid level to PUUV-N (166), between TULV-N and PUUV-N the difference is 21% (144). Thus, high levels of serological cross-reactions were expected between TOPV/TULV and PUUV. In the analyses, the rapid test was able to detect TULV- and TOPV-specific antibodies from Arvicolinace rodent blood samples. However, it is possible that the PUUV-N antigen may not provide sufficient cross-reactivity to hantaviruses carried by sigmodontine (e.g. SNV) or murine (e.g. HTNV and DOBV) rodents. These rodents are phylogenetically more distant from arvicoline rodents, and the corresponding hantaviruses are also quite distant from PUUV. SNV-N reveals a 29% paired difference to PUUV-N, whereas HTNV-N and DOBV-N show 39% difference between their N-proteins and PUUV-N (144).

With human rapid tests, the PUUV-specific IgM positive samples were found in most cases only by the PUUV rapid test. The PUUV test in turn found less than 20% of the DOBV and HTNV samples. Although cross-reactions between HTNV and DOBV were frequent, the HTNV rapid test missed one DOBV-positive serum sample. Also other studies with DOBV suggest that all the DOBV infections cannot be detected with HTNV antigen (16). Thus, this strongly recommends the use of three antigens, PUUV-N, HTNV-N and DOBV-N, in the detection of hantavirus antibodies in humans and also in rodents in the Eurasian continent.
5 Discussion

In areas like the Balkans where multiple hemorrhagic fevers occur, the differential diagnostics is an important feature in patient care. For example during an outbreak of viral hemorrhagic fevers in the Republic of Kosovo in 2001, it turned out that of the 192 suspected cases, 29 were Crimean-Congo hemorrhagic fever (CCHF) and two were HFRS cases. The clinical picture of these two diseases is similar, and the rapid laboratory confirmation of the suspected causative agent is essential for proper diagnosis (6) since the CCIIF may spread from human to human.

Furthermore, the rapid test may be used also to separate different hantavirus infections from each other. Diseases caused by PUUV and DOBV differ from each other, and this should be taken into consideration during the treatment of the patient in some cases. However, the clinical picture of PUUV and SAAV infection is quite similar, whereas SAAV and DOBV are very closely related genetically and serologically. It remains to be determined whether SAAV antibodies cross-react with the DOBV test. Since the cross-reactivity rates between PUUV and DOBV were low in the rapid test, it may be stated that the homogenous single antigen rapid tests may be used as a tool for the differentiating diagnostics of PUUV and DOBV infections.
6 FUTURE PROSPECTS

Rapid test formats will most probably gain greater and greater acceptance in the world of diagnostic tests. If the rapid test is sufficiently sensitive and specific, it helps the patient, the physician, and the laboratory technician, and serves as a cheap diagnostic method. Following the approval of a rapid HIV test kit as an official diagnostic tool for HIV infection by the FDA, the rapid tests have established a firm foothold in the field of diagnostics.

The new rapid test technology introduced in this thesis and in the original publications provides a useful platform to expand and enhance the rapid diagnostics of diseases. Studies to apply the rapid test technology also to the hantaviruses carried by the sigmodontine rodents (SNV) are in progress. Additionally, some viral and microbial infections in humans, such as tick borne encephalitis (TBE), dengue virus, Pogosta disease (Sindbis virus) and Chlamydia trachomatis bacteria are now on the way to being applied to this test technology.

The rapid test technique would also be worthwhile extending to viral infections of other animal species. If extended to other viruses carried by diverse animal vectors, this test technology may provide benefit to public health officials. It would provide a convenient tool to study the population dynamics of emerging zoonotic viruses in host animal populations.
7 REFERENCES


References


7 References


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