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Final Relation

"Evaluation of the human seroprevalence of Hantavirus infections in Italy"

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SUMMARY

Rational of the study: Hantavirus infections represent an important health problem in different parts of Europe. Unfortunately, there is still little awareness of the problem in Italy and few studies have been carried out for epidemiologic surveys in our country. The aim of this work, performed in collaboration with other 5 centers, was to establish the distribution of Hantaviruses in different Italian regions using seroepidemiological analysis. These were performed in order to put in place a series of public health and prevention strategies to contain the diffusion of this infection.

Planning of the study: This work is part of a national multicentric study involving 6 national centers (AOU “Maggiore della Carità” of Novara, “Sacco Hospital” of Milan, “Spallanzani Institute” of Rome, “Santa Maria della Misericordia Hospital” of Udine, IZTS Lazio ad Toscana "M. Aleandri" and IZTS Sicily). It is funded by the “Ministero della Salute” and approved by the Spallanzani Institute Ethical Committee. The study is still ongoing and until now the first 74 samples were included, from people that for working (farmer, forester, veterinarian...) and living condition are potentially exposed to the risk to come in contact with rodents. On all the samples, ELISA tests were performed to look for the presence of IgG and IgM against Hantaan, Puumala and Dobrava viruses. On the same samples also IFA test for the research of IgG were performed, while only 56 were tested for IgM. In both cases in IFA 6 different subtypes were evaluated: Puumala, Hantaan, Dobrava, Sin Nombre, Seoul and Saaremaa.

Results: Only two patients are positive for IgG in the ELISA assay (2.7%), including one who is strongly positive and the other who is weakly positive.
Regarding IgM in immunoenzymatic assay, 6 samples are positive (8.1%), of which 2 are high positive and 4 are low positive.
11 samples are positive for IgG detection by indirect immunofluorescence assay. 4 are positive for Saaremaa virus, 2 for Dobrava, 1 for Puumala, 1 is weak positive for Seoul,
1 low positive for Sin Nombre and 2 are weak positive for Hantaan. The overall positivity is 14.9%. However, considering only strong positives (5 out of 11), the prevalence is 6.8%.
In the immunofluorescence test for the IgM only 9 samples are positive. Among these, 8 are weak positive and 1 strong positive. Specifically, 3 are weak positive for Saaremaa, 1 is positive for Saaremaa, 3 are weak positive for Sin Nombre and the last 2 are weak positive for Puumala and Seoul respectively. The prevalence of IgM is 16.1%.
So in general 28% of the recruited patients present a positivity. It is higher respect the ones identified in previous studies performed in our country. The percentage in breeders, farmers and veterinarians is 33%, so they are more exposed respect the other subject involved in the study. We also can affirm that IFA is better respect ELISA.

Conclusions: In conclusion, although these data are still preliminary, we can assume that Hantaviruses are present in Italy with a high degree of active circulation and are increasing compared to the past. It also seems that some professions (farmers, breeders and veterinarians) are more at risk than others. Lastly, we can hypothesize the presence of Saaremaa in Italy and our study represent the first evidence of it. For the future it is important to have a continuous epidemiological surveillance, both at human and veterinary level, in order to identify the most endangered areas in the whole territory, including regions that have not been considered yet in this project.

1. GENERAL INTRODUCTION

Hantaviruses are zoonotic viruses with a nearly global distribution.[1] Their name derives from the Hantaan river in South Korea where the first member species was isolated by Karl M. Johnson e Ho-Wang Lee.[2]

Hantaviruses belong to the *Orthohantavirus* genus, family *Hantaviridae*, order *Bunyavirales*.[3] The families of this order are *Nairoviridae, Peribunyaviridae, Arenaviridae, Phenuiviridae* and others. (Fig.1)[4]

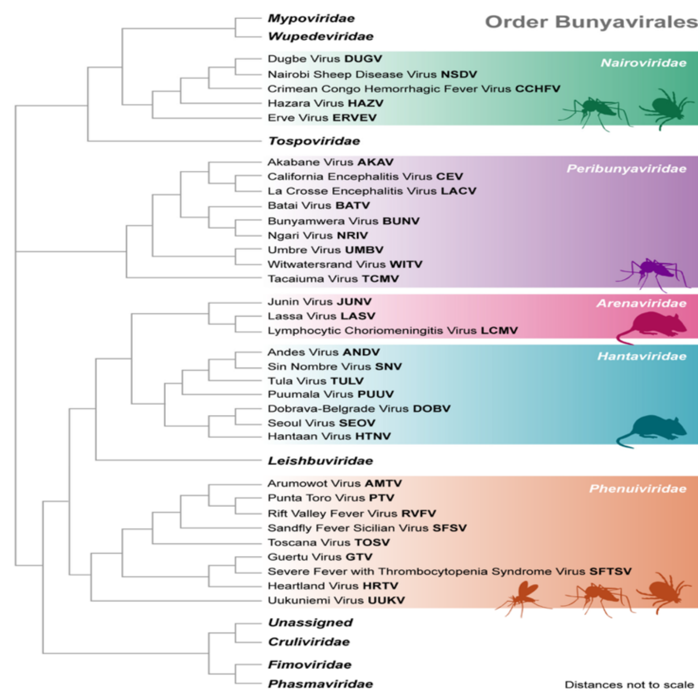

Fig.1: Bunyavirales classification.

Unlike other families of the order *Bunyavirales, Hantaviridae* are not transmitted by arthropod vectors, but directly by rodents. They are therefore not *Arbovirus*, but "*Robovirus*" (Rodent-borne viruses).[5][6]

1.1 Robovirus

A robovirus is a zoonotic virus that is transmitted by a rodent vector. The viral families Arenaviridae and Hantaviridae belong to this group.[6][7]

Rodent borne disease can be transmitted through different forms such as rodent bites, scratches, urine and saliva. Potential sites of contact with rodents include habitats such as barns, outbuildings, sheds and dense urban areas. The transmission of disease from rodents to humans can occur through direct handling and contact, or indirectly through rodents carrying the disease spread to ticks, mites, fleas.[8]
The climate conditions can influence the Roboviruses' prevalence and distribution. Warmer winters and increased rainfall increase the number of rodent reservoirs.[9]

1.2 Hantavirus

In the past century, two major outbreaks led to the discovery of Hantaviruses. The first outbreak, occurred during the Korean War, wherein more than 3,000 U.N. troops fell ill with Korean hemorrhagic fever, commonly referred to as hemorrhagic fever with renal syndrome (HFRS).[10] However, the causative agent remained unknown until the early 1980s, when Lee et al. reported on Hantaan virus (HTNV), present in the lungs of its natural reservoir, the striped field mouse (*Apodemus agrarius*).[11]

The second outbreak occurred in the Four Corners region of the U.S. in 1993 and was initially referred to as Four Corners disease. Now it is called Hantavirus Pulmonary Syndrome (HPS) or Hantavirus Cardiopulmonary Syndrome (HCPS).[10]

After these two events, more then 20 additional HFRS-related viruses were discovered in Asia, Europe and United States.[10]

During the last decade, more than 200,000 cases of hantavirus disease occur globally every year. The mortality is up to 12% (HFRS) and 40% (HCPS), depending on the species.[12]

Hantaviruses are widely endemic in Europe like the human-pathogenic Puumala and Dobrava.[13]

2. CLASSIFICATION

Hantaviruses are classified according to the geographic distribution of their hosts and the diseases they caused.

The genus *Orthohantavirus* includes at least 38 species, with more than 50 viruses. At least 18 viruses can cause human disease. All these are divided into two groups: Old and New World Orthohantaviruses (OW and NW).[14]

Despite their differences in pathogenesis, the OW and NW Hantaviruses share high homology in the organization and sequence of their genomes and exhibit similar aspects of their life cycle.[15]

Group	Virus	Rodent carrier	Disease
Old World hantaviruses	Amur/Soochong	*Apodemus peninsulae*	HFRS
	Dobrava	*Apodemus flavicollis*	HFRS
	Hantaan	*Apodemus agrarius*	HFRS
	Puumala	*Myodes glareolus*	HFRS (NE)
	Luxi	*Eothenomys miletus*	HFRS
	Saaremaa	*Apodemus agrarius*	HFRS
	Seoul	*Rattus*	HFRS
	Tula	*Microtus arvalis*	HFRS*
	Anajatuba	*Oligoryzomys fornesi*	HCPS
	Araucaria	*Oligoryzomys nigripes, Oxymycterus judex, Akodon montensis*	HCPS
	Araraquara	*Bolomys lasiurus*	HCPS
	Bayou	*Oryzomys palustris*	HCPS
	Bermejo	*Bolomys lasiurus*	HCPS
	Black Creek Canal	*Sigmodon hispidus*	HCPS
	Castelo dos sonhos	*Oligoryzomys eliurus*	HCPS
	Choclo	*Oligoryzomys fulvescens*	HCPS
	Itapua	*Oligoryzomys nigripes*	HCPS
	Juquitiba	*Oligoryzomys nigripes*	HCPS
	Laguna Negra	*Calomys laucha*	HCPS
	Lechiguanas	*Oligoryzomys flavescens*	HCPS
	Maporal	*Oligoryzomys delicatus*	HCPS
	Monongahela	*Peromyscus leucopus*	HCPS
	Neembucu	*Oligoryzomys chacoensis*	HCPS
	New York	*Peromyscus leucopus*	HCPS
	Oran	*Oligoryzomys longicaudatus*	HCPS
	Paranoa	Not known	HCPS
	Rio Mamore	*Oligoryzomys microtis*	HCPS
	Sin Nombre	*Peromyscus maniculatus*	HCPS

HCPS, hantavirus cardiopulmonary syndrome; HFRS, haemorrhagic fever with renal syndrome; NE, nephropathia epidemica.
* Association with the disease not definitely confirmed.

Fig.2: Carriers and disease associated with OW and NW Hantaviruses.

2.1 Old World

This group includes Hantavirus circulating in Europe and Asia.

They are transmitted by four kinds of rodents: Myodes, Microtus, Apodemus and Rattus, but also from two families of insectivorous: Sorcidae and Talpidae. [16]

OW Hantaviruses are responsible for the majority of notified cases; most of them occur in China as a syndrome characterized by renal failure and hemorrhagic manifestations (HFRS), with an average annual incidence of 0.83/100,000 inhabitants and a case fatality rate up to 15%.[17]

OW Hantaviruses are divided into two serogroups: those transmitted by mice and those transmitted by voles.[16]

Mouse transmitted Hantavirus

Hantaviruses transmitted by murines belong to Hantaan serogroup. These are mainly transmitted by the *Apodemus* mouse or by rat. Both are widely present in Europe.

In this serogroup are included:

Virus Hantaan (HTNT): transmitted by the *A.agrarius* mouse. It is very important for the human pathology. It is the main responsible of HFRS in Asia (mortality of 5-15%). The same mouse species can also transmit virus Amur (AMRV), Soochong (SOOV) in Asia, and the Saaremaa (SAAV) in Europe.

Dobrava-Belgrade virus (DOBV): it was isolated more than 25 years ago from a yellow-necked mouse, *Apodemus flavicollis*, in Slovenia. DOBV was then detected also in striped field mice, *A.agrarius* and in the Black Sea field mice, *A.ponticus*.[18]

Some evidence demonstrates the presence of this virus also in Italy (province of Udine).[19]

DOBV can be consider one of the most virulent European Hantavirus. It is responsible for almost all fatal HFRS cases in Europe.[18]

Virus Seoul (SEOV): transmitted by *Rattus norvegicus.* SEOV is associated with a low mortality respect the previous two. It is present in Japan, Korea but also in Europe and USA.[20]

Vole transmitted Hantavirus

The second OW serogroup is transmitted by *Myodes* voles. It is called Puumala serogroup. Voles are widely diffuse in Europe, except for Mediterranean region.

The most important virus is Puumala. It was found in bank voles (*Myodes glareolus*) in Finland in 1980.[14][16]

PUUV causes a mild form HFRS called nephropathia epidemica (NE). The mortality in this form is low (~1%). It can also cause the Guillain-Barrè syndrome. In addition, recent data provide evidence that PUUV is capable, in rare cases, of infecting the CNS.[21]

The seroprevalence is high in Northern Sweden, but Puumala was detected in the former Yugoslavia, Finland, Germany, Belgium, France, Poland.[14]

Another virus included in this serogroup is Tula virus (TULV). TULV was first isolated from common European voles (*Microtus arvalis* and *M.rossiaemeridionalis*) captured in Russia in 1987.[14]

Rarely it causes infection in human. Some cases were identified in Russia, Swiss and Germany.

2.2 New World

The NW Hantaviruses were first recognized in 1993, after an outbreak of an acute pulmonary distress syndrome in America. They are all included in a single serogroup.

The *Sigmodontinae*, a rodent family of Cricetidae, is responsible for their transmission.

They are mainly present in North and South America.[16]

Sin Nombre (SNV) belong to this group. It was first discovered in the Four Corners region of the U.S. in 1993. It is the main etiological agent of the HCPS in the U.S.

For SNV, the carrier is the common deer mouse, *Peromyscus maniculatus*. SNV is mainly present in North America, where cause different severe cardiopulmonary infections. The mortality is around the 50%.[22]

On the other hand, the most common virus in South of America is Andes virus (ANDV). It is transmitted by *Oligoryzomys flavescens.* ANDV is mainly present in Argentina and Chile, where it causes severe cases of HCPS (mortality 40-50%). Andes is the only virus for which interhuman transmission has been proven.[23]

Other viruses of this group can induce rarely human infections, like the Black Creek Canal virus in Florida, Monongahela virus (MNGV) in the USA and Canada harbored by *Peromyscus maniculatus*.

Brazil supports approximately 450 of the 540 known species of *Sigmodontinae* rodents. The virus Laguna Negra and Castelo dos Sonhos are present in this country.[10]

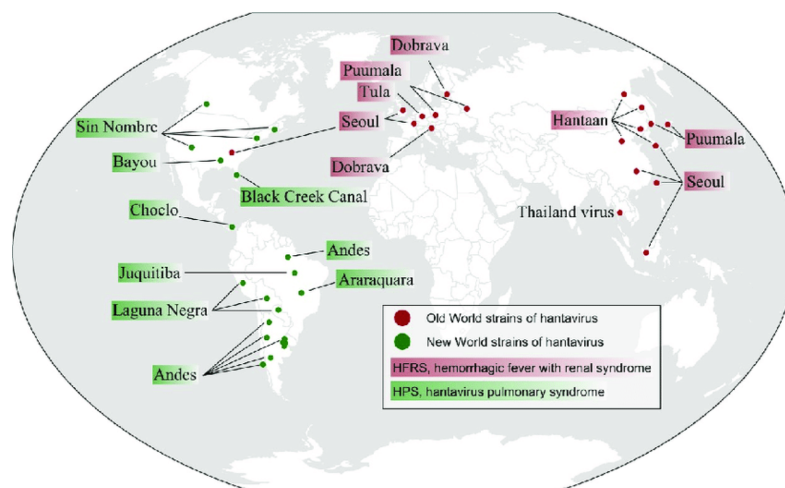

Fig.3: Geographical distribution of the main NW and OW Hantaviruses.

3. VIRION STRUCTURE AND GENOME ORGANIZATION

Hantavirus' virions are spherical with an average diameter of 80-120 nm.[24]

Like all the Bunyavirales, Hantavirus are covered by an envelope. It has a thickness of 5 nm and derives by the membrane of the Golgi apparatus.

This coating is necessary to holds the spike assemblies that protrude approximately 10 nm from the membrane and play an important role in viral entry and antibodies neutralization. The glycoprotein Gn and Gc are the main components of these protrusions. Each spike is formed by a $(Gn/Gc)_4$ tetramer with Gn at the center and Gc at the periphery.[25][26][27]

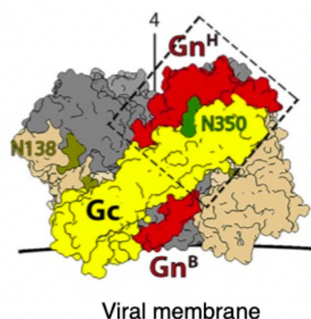

Fig.4: Representation of the spike's structure of Hantavirus.

The genome is enclosed in a spherical capsid, derived from the fusion of three different capsids inside the same envelope.[28]

The first molecular analysis of Hantaviruses showed that their genome comprises three negative-sense, single stranded RNAs that share a 3' terminal sequence of the three genome segments. [10]

These segments, classify according to their dimension, are:

- Segment S (small), it has a length of 1,8 kb and encodes for the nucleocapsids protein (N).
- Segment M (medium), it encodes for the precursor of a glycoprotein, which will be divided by a proteolytic cut to generate Gc and Gn (surface glycoproteins). It has a length of 3,7 kb.

- Segment L (large), 6,5 kb long it encodes for the protein L, the RNA dependent RNA-polymerase (RdRp).[14][22]

The total length of the genome is around 12 kb.

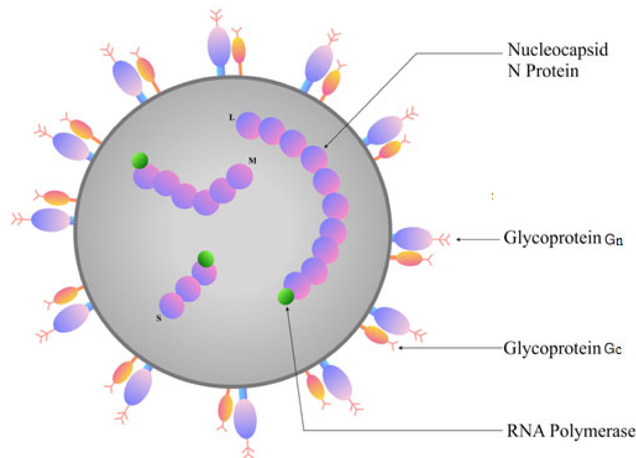

Fig.5: Virion structure of Hantavirus.

The genome of Hantavirus is highly conserved at the terminal nucleotides of the 3'-5' ends of each segment. These terminal regions harbor the capacity to form a panhandle structure, which act as the viral promoter.[26]

Hantavirus of the Hantaan and Seoul lineage do not have a nonstructural protein (NSs). However, the NW Hantaviruses and vole-borne Puumala-Tula virus contain an evolutionarily conserved NSs Open Reading Frame (ORF), similar to that of the Orthobunyaviruses.[10]

NSs in PUUV and TULV is able to inhibit the expression of ß-interferon and to interfere with the activities of nuclear factor-kB and INF-3.[29]

Hantavirus lacks of the matrix protein and so the N protein may provide this function to facilitate physical interaction between the glycoprotein on the inner leaf of the lipid membrane and the RNP. The RNP (Ribonucleoprotein complex) inside the virion consists of one viral RNA segment complexed with the N protein.[10][30]

4. REPLICATIVE CYCLE

Endothelial cells of the capillaries of lungs, kidneys, heart, liver and spleen are the main targets of Hantavirus. However also macrophages, mononuclear blood cells, dendritic cells, respiratory and tubular epithelium can be infected.[1]

The infection starts with the attachment of viral glycoprotein to the host's cell surface receptor. The receptors involved in the binding with the viral glycoprotein are integrins. β1-integrin is used by apathogenic *Microtus*-borne Hantaviruses, while the β3-integrin is used by pathogenic Hantaviruses causing HFRS and HCPS. However, these are not the unique possible receptors, because even cells without β3-integrin proteins permit infection.[10][30]

Notwithstanding, for the entry inside the cells, Hantaviruses need also a co-factor. Decay-accelerating factor (DAF)/CD55 is a GPI-anchored protein of the complement regulatory system. DAF serves as a receptor for attachment to the apical cell surface for several viruses, including Hantaan and Puumala.[31]

After the interaction with the receptor, the virion is ready to enter inside the cell. OW Hantaviruses have been shown to use clathrin-coated vesicles, while the NW use an alternative strategy, entering through endocytic vesicle and acidification of the endosome.[33]

Once the virus is inside the infected cell, it will be uncoated to release the three RNPs into the cytoplasm. The replication of these viruses is always cytoplasmatic. Hantaviruses follow the replication process used by the other RNA viruses.

Viral RdRp initiates primary transcription to give rise to the S, M and L mRNAs. The translation of the S and L mRNA transcripts occurs on free ribosomes, while the M-segment transcript occurs on membrane-bound ribosomes, which is co-translated on rough

endoplasmic reticulum. For Hantaviruses, the N protein is the most abundant viral protein and is synthesized early in infection. [34]

The Gn and Gc proteins are glycosylated in the ER and subsequently transported to the Golgi complex. The newly synthesized vRNAs are encapsulated by the N protein to form the RNPs.[10]

After budding into the Golgi apparatus, the viral particle is transported to the plasma membrane where it is then released via exocytosis.[35]

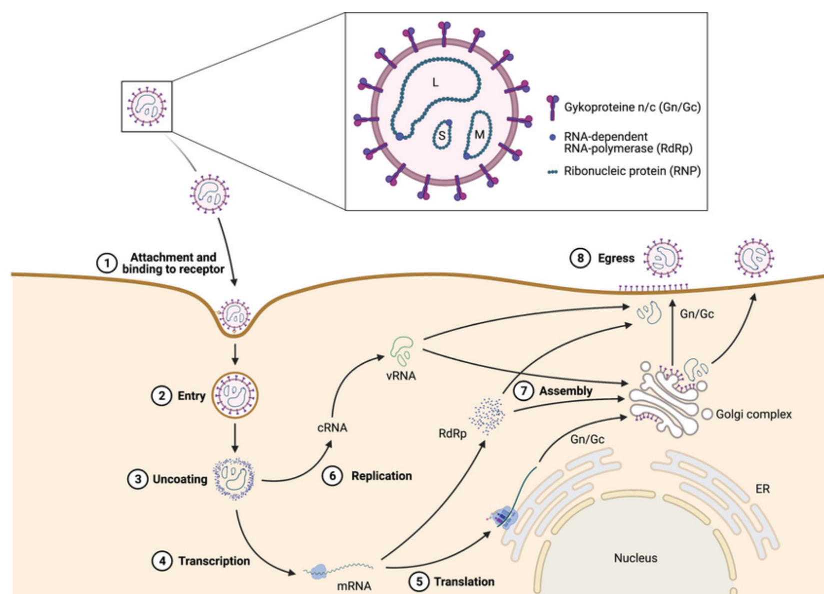

Fig.6: Replicative cycle of Hantavirus.

5. TRANSMISSION ROUTES AND PATHOGENESIS

The main host of Hantavirus is represented by small mammals.

In contrast to other Bunyaviruses, Hantaviruses are not transmitted to human by an arthropod vector, but by persistently infected rodent or insectivore hosts and even bats. [16]

In general, the infection of the natural host is inapparent and does not produce disease. Moreover, it tends to become chronic for the whole life of the animal.[36]

In nature Hantaviruses are circulating via horizontal transmission between chronically infected natural host reservoirs (mice, rats, voles).[16]

Hantavirus virions are excreted from infected rodents via saliva, urine and feces. Humans may become infected through:

- Inhalation of aerosols of dried excreta.
- Inoculation through conjunctiva.
- Entry across broken skin.
- Rodent bites.[13]

Significantly higher risk of infection was reported among forest workers, hunters, soldiers, employees on horse farm and mammologist, but also people that live in rural area.[37]

Hantavirus infection in humans can result in two clinical syndromes: Hemorrhagic fever with renal syndrome (HFRS) or Hantavirus cardiopulmonary syndrome (HCPS) caused by Old World and New World Hantaviruses, respectively. However, the majority of human infections occurs mostly unnoticed, either asymptomatic or as a mild flu-like syndrome (high fever, malaise, myalgia).

The main difference between HFRS and HCPS is based on the vascular beds affected. Renal medulla capillaries during HFRS and pulmonary capillaries during HCPS. On the other hand, the initial symptoms of all Hantavirus infections are similar, including an abrupt onset of high fever, malaise, myalgia and other flu-like symptoms.[38]

Increased vascular permeability is central to pathogenesis. This is not caused by a lytic effect of the virus. It could be triggered by binding of the virus to cell receptors that regulate

endothelial permeability, increased innate immune responses and inflammatory response.[39]

Hemorrhagic fever with renal syndrome (HFRS)

The clinical presentation of HFRS depend in part on the causative agent of the disease. In general, HFRS caused by HTNV, Amur/Soochong virus or DOBV are more severe (mortality 5-10%), whereas SEOV causes moderate disease and PUUV and SAAV cause mild diseases (mortality <1%). [16]

Symptoms of HFRS usually develop within one to two weeks after exposure, but in rare cases, they may take up to eight weeks to develop. HFRS outbreaks are more frequent in winter and early spring.[24]

The course of the illness can be split into five phases(Fig. 7):

- Febrile phase: it lasts 7 days and is accompanied by headache, abdominal pain, nausea, coagulation abnormalities, thrombocytopenia, and hemorrhagic diatheses. At the end it is possible to manifest proteinuria.
- Hypotensive phase: it can last from hours to two days. Thrombocytopenia and leukocytosis are characteristic of this phase. In severe cases, hypotension, even shock, may develop rapidly and one-third of HFRS deaths are associated with fulminant irreversible shock at this stage.
- Oliguric phase: it lasts 1-16 days and can be associated with oliguria (<400ml/day), hypertension, pulmonary edema and complications of kidney function. In this phase patient can be treated with hemodialysis. One-half of fatalities occur during this phase.

- Diuretic phase: it can last for days or weeks. It is a positive prognostic sign for the patient. Further clinical problems may be dehydration, electrolyte shifts and secondary infections.
- Convalescence phase: lasting 2-3 months, usually associated with complete recovery.[10][16]

In milder forms of HFRS, the five phases are not easily distinguishable. The severe form induce by DOBV follow this course, with core complications and shock(21-28%).[16]

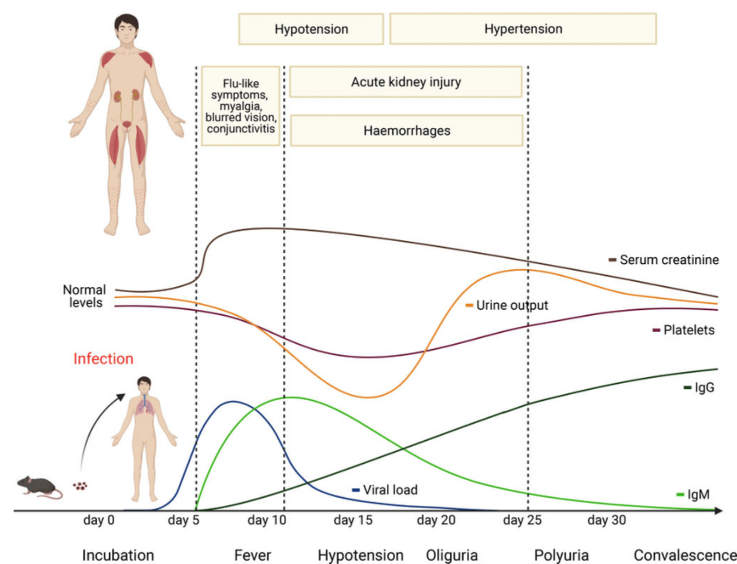

Fig.7: Typical course of HFRS in human.

Nephropathia epidemica (NE) was first described in Sweden in the 1930s and thousands of Hantavirus infection cases occur annually throughout Europe.

Puumala virus is by far the most prevalent pathogen. NE is a mild form of HFRS that is characterized by acute kidney injury (AKI) and thrombocytopenia. The occurrence of thrombocytopenia in infected patients varies from 39% to 98%.[24][41]

Hantavirus cardiopulmonary syndrome (HCPS) was first described in the U.S. in 1993. The etiological agents are Sin Nombre and Andes viruses, mainly present in North and South America respectively.[24]

Cases of HCPS have been reported for the following countries: United States, Canada, Argentina, Bolivia, Brazil, Chile, Panama, Paraguay and Uruguay.[10]

HCPS-causing Hantaviruses mainly target the respiratory and cardiovascular systems. It is more severe respect HFRS and the mortality rate is between 30%-50%.[43]

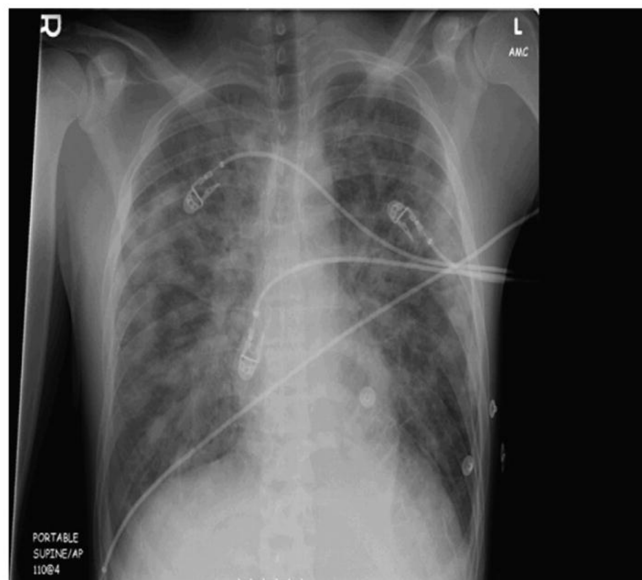

Figure 8: Chest radiograph of a patient in the cardiopulmonary phase showing diffuse alveolar filling and small bilateral pleural effusions.

6. IMMUNOLOGY

The wide spectrum of disease observed among hantavirus-infected patients might be related to differing immune responses and viral load kinetics.

DOBV-infected patients were found to have a higher viral load than the PUUV-infected patients (10^7 vs. 10^5 RNA copies/mL).

The virus has no direct cytopathic effect on primary target cells. The host inflammatory response seems to play an important role in the pathogenesis. Hantavirus-infected patients exhibit an elevated proinflammatory cytokine profile in the serum, organs and tissues (TNF-α/IL-6/IL-2/IL-1/IL-10). However also the hantavirus-specific CD4+ and CD8+ T cells contribute to the cytokine storm and capillary leak, with the consequent pulmonary edema and cardiogenic shock during HCPS.[45][46](Fig.9)

In the first 8-25 days of infection antibodies of the class IgM are produced. They indicate the presence of an acute infection. IgG can be identified in the 60% of the cases of HFRS since the 14th day past infection.[47]

nAbs have been detected also years after PUUV, SNV and ANDV infections, implying a long-lasting immune response.[46]

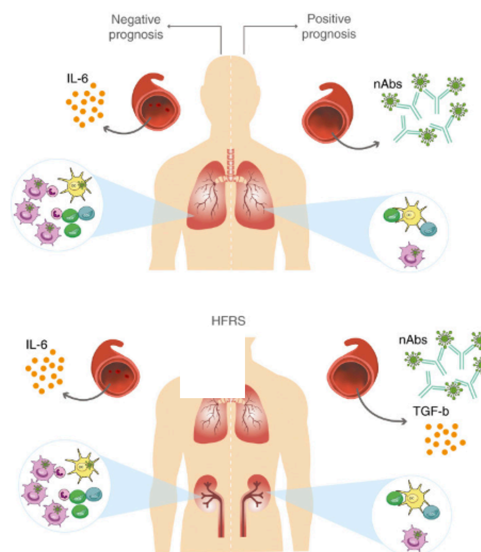

Figure 9: Immune mechanisms in Hantavirus prognosis disease.

7. LABORATORY DIAGNOSIS

The diagnosis of Hantavirus infections in humans is based on clinical and epidemiological information, as well as laboratory tests.[51]

The symptoms that should alert the physician to a possible Hantavirus infection are high fever, headache, abdominal and back pains. Among the pathological laboratory findings leukocytosis, thrombocytopenia, increased serum creatinine, proteinuria and hematuria are present.[1]

However, it is almost impossible to diagnose Hantavirus infections solely on clinical grounds, especially in cases with mild clinical symptoms, as the early signs of the disease are non-specific.[11]

Serological diagnosis

Serology is the most widely used diagnostic test. Virtually all acute HFRS and HCPS cases have IgM and IgG antibodies against the N protein. One of the first serological tests used for diagnoses of HFRS was the indirect immunofluorescence assay(IFA) using hantavirus-infected cells fixed as an antigen on microscope slides.[10]

The most utilized serological tests are indirect IgM and IgG ELISA as well as IgM capture ELISAs, which have higher specificity than indirect ELISAs. The speed of execution of ELISA is a benefit; the results can be accessed within 4 hours.[11]

All these assays are based on viral antigens expressed in infected cells. The structural proteins (Gn,Gc and N) can induce a high level of IgM at the onset of symptoms. The IgG response to the glycoproteins may be delayed, and in the acute phase, the diagnostic IgG IFA pattern is granular.[52]

Also, an immunoblot assay, based on the use of N antigen, has been established. Proteins from whole-cell extracts are used.[52]

In certain cases, ELISA, IFA or immunoblot assays were unable to support the diagnosis of PUUV infection because of extended cross-reaction of IgM and even IgG antibodies to other Hantavirus antigens. In these cases, focus reduction neutralization testing (FRNT) confirmed PUUV infection. [53]

Molecular diagnosis

The Hantavirus infection can be confirmed by detection of viral genome by Reverse transcription polymerase chain reaction (RT-PCR), starting from different samples like blood and organ fragment. In this way, the genome can be identified from the first day after the onset of illness.[1]

Although the presence of viraemia varies, viral RNA can usually be detected if an acute sample is available. It has also been suggested that higher viraemia is found in more severe Hantavirus infections (DOBV, SNV, ANDV), compared with milder infections, caused by PUUV.[11]

In a study, it was possible to determine that RT-qPCR had a low detection limit (~10 copies), with high specificity (100%) and sensitivity (94.9%). This suggests the potential for establishing RT-qPCR as the assay of choice for early diagnosis, promoting early effective patients' care.[54]

8. TREATMENT

The management of severe cases is purely based on supportive care. Maintaining fluid and electrolyte balance is very important. Oxygen administration is required in many cases.

HFRS patients with severe renal insufficiency may need extracorporeal blood purification (dialysis treatment). In HCPS, mechanical ventilation or even extracorporeal membrane oxygenation may be required.

At present, trial of antiviral and immunotherapies against HFRS and HCPS have been performed.

One of this is Ribavirin. It has been confirmed that administration of intravenous Ribavirin early in the course of HFRS reduces the occurrence of oliguria and the severity of renal insufficiency.[11]

Another is Favipiravir, it was effective in ANDV and SNV animal models when given before onset of viraemia. Icatibant Acetate, a bradykinin receptor antagonist, has been used in several patients with severe HFRS.[1]

Also immunotherapy is tested recently. Recombinant monoclonal antibodies have been developed from isolated memory B cells. The resulting monoclonal antibody candidates, JL16 and MIB22, had been shown to effectively neutralize ANDV in vitro.[55]

9. PREVENTION

The preventive measures are based mainly on rodent control, reducing rodents' shelter and food sources near human housing, eliminating rodents inside homes and avoiding contact with potentially contaminated areas. Apart from using standard precaution measures, the only way of minimizing the risk of hantavirus disease could be effective vaccines. Up to now no vaccines were approved for wide use in Europe and USA.[55]

10. EPIDEMIOLOGY

The epidemiology of human Hantavirus infections is based on incidences of peridomestic exposure of humans to rodents in areas of endemicity. The time and space distribution of Hantavirus infections in men mirror the distribution of their rodent hosts.[10]

The total number of Hantavirus case reports is generally on rise, as is the number of affected countries. Climatic, ecological, and environmental changes are related to fluctuations in rodent populations and subsequently to human epidemics.

Currently, it is estimated that 150,000-200,000 cases of hantavirus disease occur per year, of which 70%-90% correspond to HFRS cases in China. However, reported cases in the known endemic areas in Asia, Scandinavia and Americas seem to be on the rise.[59]

Initially, it was believed that HFRS occurred only in rural areas of Eurasia, specifically China, South Korea, eastern Russia and northern Europe. Prudent surveillance demonstrated that HFRS caused by SEOV could also occur in urbanized cities and in many parts of the world. Cases of HFRS have been identified in Europe and Asia. On the other hand, HCPS cases have been described only in the Americas. (Fig.10)[60]

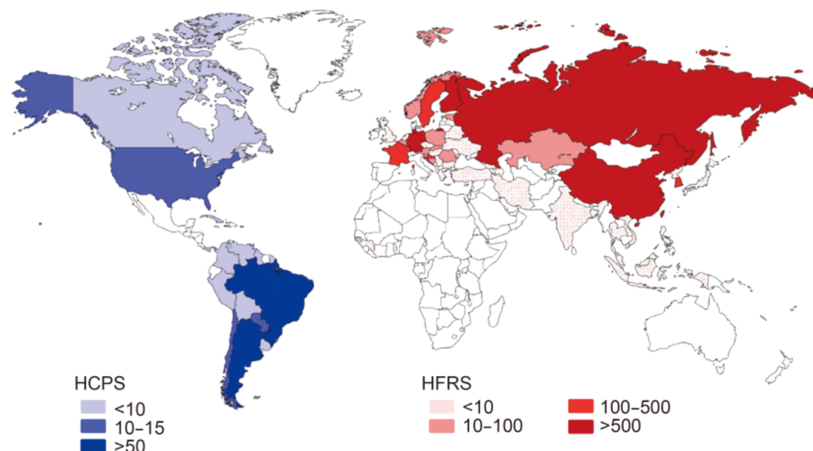

Fig.10: Geographical representation of the incidence of HCPS and HFRS by country per year (2016).

In China, HFRS is considered a severe public health challenge with 90% of the total reported cases in the world. Here HFRS cases occurred most frequently in June, November and December. HNTV and SEOV are the major causes.[61]

Asian-Russia is another area affected by Hantavirus infections. Indeed, this region accounted for 3,145 HFRS cases. Viruses in Asian-Russia show great similarity to those in China and Korea, and PUUV has been found in Far-Eastern-Russia.

In Korea, where HTNV was first isolated, 300-500 HFRS cases are reported annually (mean case fatality rate of 1%). Farmers account for the largest proportion of HFRS (35.6%) cases, most of which were detected during October, November and December. However, in Korea also SEOV was identified.

Serological evidence of Hantavirus infections in humans, particularly in patients with fever of unknown origin, has been reported in Vietnam, Singapore, Laos, Thailand, Malaysia, Indonesia, India and Sri Lanka. [24][62]

Before 2006 no indigenous African Hantaviruses were identified. Within recent years, new discoveries have been made and over 10 Hantaviruses have now been identified in Africa. [63]

Epidemiology in Europe and Italy

Recent epidemiological studies have shown that these viruses are more widespread in Europe than previously thought and could be the cause of many human infections.

Over 3,000 HFRS cases are diagnosed annually in Europe. The majority of infections are causes by Puumala. However, also the Dobrava has been reported in at least 10 European countries, essentially in the Balkan area.[64]

Most cases of PUUV infections in Europe come from Finland(24,672 cases before 2007). More than a thousand cases have been recorded in Sweden, Belgium, France, Germany and Norway. In northern Europe, the epidemics typically peak in November and December during high-rodent-density years, with another peak in August.

In Finland and northern Sweden, the average seroprevalence is about 5%. In Germany the seroprevalence in the endemic area can reach the 3%.

The incidence of HFRS in Russia varies geographically. The disease is most endemic in regions of the Volga river where PUUV infection prevails. In the Central Federal District in western Russia a SAAV outbreak has occurred.

Nowadays, some studies, based on the seroprevalence, show the presence of Hantavirus also in Italy. However, there is a lack of knowledge and awareness about the Hantavirus infections in Italy.

Until now different studies have been performed to understand better the distribution of Hantaviruses in our country.

The first one was performed in 2006 to evaluate the spatial and temporal distribution of hantavirus rodents in Trentino. In this study Kallio-Kokko et al, research the presence of anti-hantavirus antibodies in human and rodents.

From here antibodies to DOBV were found for the first time in Italy in the respective carrier host species (*A.flavicollis)* suggesting that DOBV is circulating in the Trentino region. In addition, DOBV IgG antibodies were detected in a human with a potential professional exposure in the same region, with a seroprevalence of 0,2%. Since DOBV infection is more severe than PUUV infection, further studies on the distribution and prevalence of DOBV in other areas in Italy are warranted to assess the public health significance of this finding.[65]

A second study was done in central Italy by Cosseddu et al, in 2017. The aim was to monitor the circulation of PUUV, DOBV, WNV and USUV in natural environment using serological surveillance in wild rodents. During this study, 90 animals were captured in Abruzzo and Marche regions and tested with serological assays for the specific pathogens. Serological tests provided no evidence of PUUV and DOBV circulation in the studied area.[66]

However, other studies have been performed in Northern Italy. In 2019 a study conducted in Piedmont (Novara and surrounding provinces) by Faolotto et al. demonstrated that on 371 samples, collected in the general population, the 2% were seropositive for DOBV and the 7% for PUUV. This work was performed at the ‘Maggiore Hospital della Carità" in Novara, specifically in the laboratory of Microbiology and Virology.

Again, in 2015 Tagliapietra et al, demonstrated that, in the autonomous province of Trento, the seroprevalence of PUUV between forester was 10%, while in the general population was 4%.[67]

The previous two works (2006 and 2015) showed an increase of Hantavirus in Trentino that can make us think to an increase of hantavirus diffusion in our country. However, available studies were performed only in Trentino and Piedmont, so other works are necessary to evaluate their presence in other Italian regions.
This means that these viruses are actively circulating in our country. As result, some other studies are necessary to evaluate their role in the Italian public health and to take specific decision for the management of this health problem.

THE OBJECTIVE OF THE THESIS

As described in the introductory section, Hantavirus infections represent an important health problem in different parts of Europe. Unfortunately, there is still little awareness of the problem in Italy and few studies have been carried out for epidemiologic surveys in our country. However, the few conducted studies seem to indicate a high circulation of these pathogens in some regions of Italy.

To clarify the epidemiological distribution in Italy, a working group consisting of 6 national centers was formed (AOU "Maggiore della Carità" of Novara, "Sacco Hospital" of Milan, "Spallanzani Institute" of Rome, "Santa Maria della Misericordia Hospital" of Udine, IZTS Lazio and Toscana "M. Aleandri" and IZTS Sicily). These entities are in the process of carrying out seroepidemiological analyses in the different areas of responsibility, in order to define a national distribution of the presence of these viruses.

By better defining the geographic distribution and epidemiology of Hantaviruses, it will also be possible to define the real importance of these infections in our country and consequently involve clinicians with the aim of improving the management of potentially infected individuals. This work also aims to define possible conditions associated with increased population risk of becoming infected with Hantavirus like the lifestyle, working conditions, animal contact and the type of residence environment (rural).

MATERIAL AND METHODS

This work, as explained earlier, is part of a national multicentric study involving 6 national centers (AOU “Maggiore della Carità” of Novara, “Sacco Hospital” of Milan, “Spallanzani Institute” of Rome, “Santa Maria della Misericordia Hospital” of Udine, IZTS Lazio and Toscana "M. Aleandri" and IZTS Sicily). The study, which is still ongoing, is funded by Italian “Ministero della Salute” and is scheduled for completion in May 2025.

The project *“Sviluppo di un sistema di sorveglianza integrata dell’infezione da Hantavirus in Italia con un approccio One Health”* has been approved under the CUP F43C22000330001.
It also underwent to the Spallanzani Institute Ethical Committee approval (9-2023 17/07/2023) and was conducted in strict accordance with the Declaration of Helsinki.

The six participating centers are responsible for collecting both human and animal (rodent and bat) samples and analyzing them first serologically and then molecularly. In particular, animal samples are tested by the two zooprophylactic institutes, while human samples are tested by the remaining 4 clinical centers.

The same serologic and molecular diagnostic kits are used in all clinical centers. In addition, the “Spallanzani Institute” in Rome will also carry out metagenomic testing. The plan is to collect, in each center, a minimum of 140 samples from as many patients considered at risk for Hantavirus infection. So, the final goal will be reach a total of 840 samples.

1. Patients involve in the study

In this thesis we show the partial results, of the still ongoing project, collected and analyzed at the "AOU Maggiore della Carità" of Novara.

In this study were included only people that for working (farmer, forester, veterinarian...) and living condition are potentially expose to the risk to come in contact with rodents directly or with their excrement.

All the patients are voluntaries in healthy condition. Each one signed an inform consent and a data collection form at the time of the sampling.

As mentioned above, the study is still ongoing, but as of May 2024, 74 patients have been recruited out of the total 140 planned. Specifically, samples were collected from December 2023 to May 2024.

The selection process involved sampling patients from different centers mainly in Novara, VCO, Vercelli, Biella and areas that are adjacent to these four provinces. The sampling has been done in our center in the Novara Hospital and in other available facilities.

In the following table are represented the main characteristics of the patient involved so far in the study. (Table 1)

The average age is 45,85 years old (range 9-85 y.o.). 56,8% of the patients were male (42) and 43,2% female (32).

On the total, 24 patients had a job with a high risk to come in contact with rodents (farmer, veterinarians and breeders). However, more in general 37 patients declared that they were exposed to risk of contacting rodents during their working activity (also different from the previous three). Lastly, we consider also the possibility to come in contact with rodents during activity of the daily life (sport, garden activity and so on...). In this class were included 32 patients.

CHARACTERISTICS OF PATIENTS (n. 74)		
Age	Range (min;max)	9;85
	Mean	45,85
Sex (n,%)	M	42 (56,8%)
	F	32 (43,2%)
Working condition	Farmers	17 (23,6%)
	Breeders	3 (4,2%)
	Veterinarians	4 (5,6%)
	Others*	48 (66,6%)
	Not specified	2
Living condition (house in the country)	Yes	52 (74,3%)
	No	18 (25,7%)
	Not specified	4
Rodents exposure during working activity	Yes	37 (56,9%)
	No	28 (43,1%)
	Not specified	9
Probably rodents exposure during activities of daily living*	Yes	32 (45%)
	No	39 (55%)
	Not specified	3

Table 1: Patients' characteristics. (*others: retiree, students, employee. *activities of daily living: trekking, garden, sport)

The patients were found to reside in various areas both rural and urban in the provinces of Novara (27), Vercelli (7), Biella (26) and VCO (3), or in areas adjacent to these 4 provinces, in the provinces of Pavia, Milan, Alessandria, and Turin (11). 74,3% declared to live in rural area and the same proportion lives with domestic animals.

Figure 11: Main residence' provinces of the patients.

Conservation and pre-treatment of samples

Blood samples for testing the seroprevalence were obtained by peripheral blood collection using two different tubes. The first without anticoagulant for the research of antibodies in the serum. The second containing the anticoagulant K2-EDTA was used to obtain the plasma, eventually analyzed to look for the viral load by a PCR test.

Within 2 hours from the collection, both the tubes were centrifugated (3000 rpm for 20 minutes) and then separated from the corpuscular part to obtain 2 aliquots of 1 ml of both serum and plasma. In this way all the samples were stored at -80°C until the analysis moment.

The samples collected were analyzed for the presence of IgG and IgM by the ELISA and IFA (ImmunoFluorescent assay) methods only. Subsequent analysis by western blot and eventual Real-Time PCR amplification have been planned for all positive cases. All PCR-positive cases will be send to the "Spallanzani Institute" for metagenomic analysis.

2. Laboratory tests

Enzime-Linked Immunoasorbent Assay (ELISA)

As first level analysis, we looked for the presence of IgG and IgM with an Enzime-Linked Immunoasorbent Assay (ELISA). It was done on each serum sample.

To perform this test the kits used are Anti-Hantavirus Pool 1 "Eurasia" ELISA (IgM or IgG) EUROIMMUN (EI 278h-9601-1 G / M).

These kits contain a mix of recombinant nucleocapsid antigens fixed inside a 96-Wells-plate. In this way is possible to diagnosticate, in a semiquantitative way, infections caused by the viral strains: Hantaan, Dobrava and Puumala.

Procedure:

The first step consists in the dilution of the samples. For the IgG research serums were diluted 1:101 using the dilution buffer. While for the IgM, the same dilution was reached adding to each sample another buffer containing a solution of goat antibodies against human IgG. Along this line, is possible to prevent false positive results caused by the rheumatoid factor.

Starting from the first well of the plate, we left the first empty (blank) necessary for the interpretation of the result. From the second one (B1) we transfer 100 µl of the calibrator, of positive and negative control and of each sample respectively (as represented in the Figure 12). For IgM semiquantitative research was used the Calibrator 2, while for the IgM only one calibrator was present inside the kit. After the dispensation, we cover and put the plate inside a thermostat for 60 minutes (+37°C ± 1°C).

	1	2	3	4	5	6	7	8	9	10	11	12
A	B	S 5										
B	Cal	S 6										
C	C +	S 7										
D	C -	S 8										
E	S 1	S 9										
F	S 2	S 10										
G	S 3	S 11										
H	S 4	S 12										

Fig.12: ELISA' plate schematic representation.

After 60 minutes, all the wells were washed using 300 µl of a specific washing buffer 10x (diluted with distilled water). Wash was repeated for three time.

In the next phase, the enzyme conjugated was added starting from the second well (Abs against human IgG or IgM conjugate with peroxidase). Another incubation of 30 minutes at room temperature was necessary to allow the conjugate to bind antibodies present in the samples. Other 3 washes were performed and then 100 µl of substrate was added starting

from A1. At this point, the plate was incubated for 15 minutes at room temperature in dark condition. As last step, the stop solution was deposited in each wells and the reading was performed. The stop solution consists of sulfuric acid that blocks the enzymatic reaction. For the reading is required a wavelength of 450 nm. To do that we used the BioTek EI x800 absorbance reader connected to the Microline 3320 9 Pin printer. (Fig.13)

Fig.13: BioTek EI x800.

To calculate the result, it is necessary to divide the sample absorbance value with the calibrator value.

Ratio <0,8:	**negative**
Ratio between 0,8 and 1,1:	**borderline**
Ratio ≥ 1,1:	**positive**

Indirect Immunofluorescence Assay (IFA)

Even in this case, the serum samples were analyzed with an IFA assay. This indirect immunofluorescence assay is based on the use of Hantaviruses' infected cells. (Fig.14)

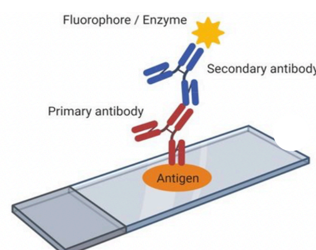

Fig.14: IFA for Hantavirus.

To perform this test the kit Hantavirus Mosaic 1 of EUROIMMUN was used (FR 278h-1005-1 G for IgG and FR 278h-1005-1 M for IgM).

Each slide contains 10x6 BIOCHIP coaptated with 6 types of Hantavirus infected cells (Hantaan, Puumala, Seoul, Saaremaa, Dobrava and Sin Nombre). (Fig.15)

		Hantavirus Mosaic 1 FI 278h-10101-1 G o M
Pozzetti 1-10	1	Hantavirus Hantaan (HTNV)
	2	Hantavirus Puumala (PUUV)
	3	Hantavirus Seoul (SEOV)
	4	Hantavirus Saarema (SAAV)
	5	Hantavirus Dobrava (DOBV)
	6	Hantavirus Sin Nombre (SNV)

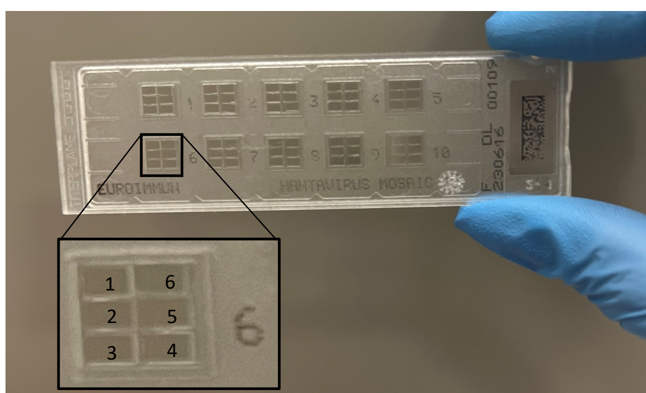

Fig.15: Slide for Hantavirus' IFA.

Procedure:

All the serum were diluted 1:100. Two different methods were used for the research of IgM or IgG. In the first case, the serum sample was diluted 1:10 using Eurosorb and incubated for 15 minutes at room temperature (10 µl of serum+ 90 µl of EUROSORB). This is done with the aim to prevent IgM false positive results and to remove the rheumatoid factor. After the incubation, another 1:10 dilution was performed (10 µl of pre-diluted serum + 90 µl of the sample buffer). In this way the final dilution was 1:100.

More easy was the dilution of the sample for the IgG. In this case 90 µl of the sample buffer was added to 10 µl of the serum, and again 90 µl of the same buffer was added to 10 µl of the previous diluted sample.

Once the samples were ready, 30 µl of the positive control, negative control and of all the sample were pipetted on a specific glass support. Then the reaction started when the slide

was placed on the support containing the reagents. The slide was incubated for 30 minutes at room temperature.

After the incubation, a washing was performed. To do that the slide was dipped in a cuvette containing PBS-tween for 5 minutes.

The next phase consisted in the deposition on the support of 25 μl antibodies anti-human IgG or IgM conjugated with fluoresceine. At the end of the wash, the slide was dried and then put on the support with the conjugate. Another incubation of 30 minutes was necessary. From this point the slide couldn't be exposed to the direct light, that will degrade the fluorescence.

In the last phase, after another wash was necessary to assemble the slide. To do that 10 μl glycerin was deposed on the coverslip, on which the dry slide have been put.

In this way the slide was ready to be read with the fluorescence microscope LEICA DM4500B using a 400x magnification. (Fig.16)

The antibodies against Hantavirus determined a cytoplasmatic fluorescence with very tiny drops. For all the cases some photos have been acquired using the Leica IMS500 Interactive Microscopy System.

Fig.16: Leica DM4500B.

3. Statistical Analysis

A final statistical analysis has not yet been carried out. However, for method comparison, the program easy Fisher Exact Test Calculator was used **(**Social Science Statistics, www.socscistatistics.com).

The definitive statistical report will be performed at the end of the study by the epidemiological center of the “Spallanzani Institute” in Rome. Here hantavirus’ prevalence will be estimated with corresponding 95% confidence interval (α=0,05), while the quantitative variables will be synthetized as mean and standard deviation or median and interquartile range, according to their distribution.

The t-test or Mann-Whitney test will be used to compare quantitative variables. While the comparison between the proportions will be done by using the chi-square test or the Fisher's exact test.

RESULTS

At the end of June, ELISA tests for the research of IgG and IgM were performed on all 74 patients enrolled in the study. Also, the IgG research using IFA test was done on all the samples. Whereas only 56 patients have been tested with indirect immunofluorescence assay to look for IgM.

However, analyses are ongoing for those that have not been tested yet.

In the coming months the study is expected to be completed, until the 140 samples planned for the Novara center are achieved.

This thesis presents the results obtained from the tests carried out at the Hospital "Maggiore della Carità" of Novara. The subsequent national processing, including the data of the other 5 centers participating in the study, will be carried out after all the expected results have been obtained.

The table below shows the results in relation to the 74 patients who have been enrolled in Novara so far. (Table 2)

SAMPLES	RESULTS ELISA IgG	RESULTS ELISA IgM	RESULTS IFA IgG	NOTES	RESULTS IFA IgM
U1-NO	NEGATIVE	NEGATIVE	NEGATIVE		UNEXECUTED
U2-NO	NEGATIVE	NEGATIVE	NEGATIVE		UNEXECUTED
U3-NO	NEGATIVE	NEGATIVE	NEGATIVE		UNEXECUTED
U4-NO	NEGATIVE	NEGATIVE	NEGATIVE		UNEXECUTED
U5-NO	NEGATIVE	NEGATIVE	NEGATIVE		UNEXECUTED
U6-NO	NEGATIVE	NEGATIVE	NEGATIVE		UNEXECUTED
U7-NO	NEGATIVE	NEGATIVE	NEGATIVE		UNEXECUTED
U8-NO	NEGATIVE	NEGATIVE	NEGATIVE		UNEXECUTED
U9-NO	NEGATIVE	NEGATIVE	NEGATIVE		UNEXECUTED
U10-NO	NEGATIVE	NEGATIVE	NEGATIVE		UNEXECUTED
U11-NO	NEGATIVE	NEGATIVE	NEGATIVE		UNEXECUTED
U12-NO	**POSITIVE**	NEGATIVE	**POSITIVE (SAAV)**	**WEAK POSITIVE (PUUV)**	UNEXECUTED
U13-NO	NEGATIVE	NEGATIVE	**POSITIVE (SAAV)**	**WEAK POSITIVE (SEOV, HTNV)**	UNEXECUTED
U14-NO	NEGATIVE	NEGATIVE	NEGATIVE		UNEXECUTED
U15-NO	NEGATIVE	NEGATIVE	NEGATIVE		UNEXECUTED
U16-NO	NEGATIVE	NEGATIVE	NEGATIVE		UNEXECUTED
U17-NO	NEGATIVE	NEGATIVE	NEGATIVE		UNEXECUTED
U18-NO	NEGATIVE	NEGATIVE	NEGATIVE		UNEXECUTED
U19-NO	NEGATIVE	NEGATIVE	NEGATIVE		NEGATIVE
U20-NO	NEGATIVE	NEGATIVE	NEGATIVE		NEGATIVE
U21-NO	NEGATIVE	NEGATIVE	NEGATIVE		NEGATIVE
U22-NO	NEGATIVE	NEGATIVE	NEGATIVE		NEGATIVE
U23-NO	NEGATIVE	NEGATIVE	NEGATIVE		NEGATIVE
U24-NO	NEGATIVE	NEGATIVE	NEGATIVE		NEGATIVE

U25-NO	NEGATIVE	NEGATIVE	NEGATIVE		NEGATIVE
U26-NO	NEGATIVE	NEGATIVE	NEGATIVE		NEGATIVE
U27-NO	NEGATIVE	NEGATIVE	NEGATIVE		NEGATIVE
U28-NO	NEGATIVE	**POSITIVE**	**POSITIVE (DOBV)**	**WEAK POSITIVE (SEOV, HTNV)**	NEGATIVE
U29-NO	NEGATIVE	NEGATIVE	NEGATIVE		NEGATIVE
U30-NO	NEGATIVE	NEGATIVE	NEGATIVE		NEGATIVE
U31-NO	NEGATIVE	NEGATIVE	**POSITIVE (PUUV)**	**WEAK POSITIVE (SEOV)**	NEGATIVE
U32-NO	NEGATIVE	NEGATIVE	NEGATIVE		NEGATIVE
U33-NO	NEGATIVE	NEGATIVE	NEGATIVE		NEGATIVE
U34-NO	NEGATIVE	**POSITIVE**	NEGATIVE		NEGATIVE
U35-NO	**WEAK POSITIVE**	**WEAK POSITIVE**	**WEAK POSITIVE (HTNV)**		NEGATIVE
U36-NO	NEGATIVE	NEGATIVE	NEGATIVE		**WEAK POSITIVE (SAAV)**
U37-NO	NEGATIVE	NEGATIVE	NEGATIVE		NEGATIVE
U38-NO	NEGATIVE	NEGATIVE	NEGATIVE		**WEAK POSITIVE (SAAV)**
U39-NO	NEGATIVE	NEGATIVE	NEGATIVE		NEGATIVE
U40-NO	NEGATIVE	NEGATIVE	NEGATIVE		NEGATIVE
U41-NO	NEGATIVE	NEGATIVE	**WEAK POSITIVE (HTNV)**		**WEAK POSITIVE (SAAV)**
U42-NO	NEGATIVE	NEGATIVE	NEGATIVE		**WEAK POSITIVE (SNV)**
U43-NO	NEGATIVE	NEGATIVE	**WEAK POSITIVE (SNV)**		NEGATIVE
U44-NO	NEGATIVE	NEGATIVE	NEGATIVE		NEGATIVE
U45-NO	NEGATIVE	NEGATIVE	NEGATIVE		NEGATIVE
U46-NO	NEGATIVE	NEGATIVE	NEGATIVE		NEGATIVE
U47-NO	NEGATIVE	NEGATIVE	NEGATIVE		**WEAK POSITIVE (SNV)**
U48-NO	NEGATIVE	**WEAK POSITIVE**	NEGATIVE		**WEAK POSITIVE (SEOV)**
U49-NO	NEGATIVE	NEGATIVE	NEGATIVE		**WEAK POSITIVE (PUUV)**
U50-NO	NEGATIVE	NEGATIVE	NEGATIVE		NEGATIVE
U51-NO	NEGATIVE	NEGATIVE	NEGATIVE		NEGATIVE
U52-NO	NEGATIVE	NEGATIVE	NEGATIVE		NEGATIVE
U53-NO	NEGATIVE	NEGATIVE	NEGATIVE		NEGATIVE
U54-NO	NEGATIVE	NEGATIVE	NEGATIVE		NEGATIVE
U55-NO	NEGATIVE	**WEAK POSITIVE**	NEGATIVE		NEGATIVE
U56-NO	NEGATIVE	NEGATIVE	**WEAK POSITIVE (DOBV)**	**WEAK POSITIVE (PUUV)**	NEGATIVE
U57-NO	NEGATIVE	NEGATIVE	NEGATIVE		NEGATIVE
U58-NO	NEGATIVE	NEGATIVE	NEGATIVE		NEGATIVE
U59-NO	NEGATIVE	NEGATIVE	NEGATIVE		NEGATIVE
U60-NO	NEGATIVE	NEGATIVE	NEGATIVE		NEGATIVE
U61-NO	NEGATIVE	NEGATIVE	NEGATIVE		**WEAK POSITIVE (SNV)**
U62-NO	NEGATIVE	NEGATIVE	NEGATIVE		NEGATIVE
U63-NO	NEGATIVE	**WEAK POSITIVE**	**POSITIVE (SAAV)**	**WEAK POSITIVE (ALL OTHER SPOTS)**	NEGATIVE
U64-NO	NEGATIVE	NEGATIVE	**LOW POSTIVE (SEOV)**		NEGATIVE
U65-NO	NEGATIVE	NEGATIVE	NEGATIVE		**POSITIVE (SAAV)**
U66-NO	NEGATIVE	NEGATIVE	NEGATIVE		NEGATIVE
U67-NO	NEGATIVE	NEGATIVE	**WEAK POSITIVE (SAAV)**		NEGATIVE
U68-NO	NEGATIVE	NEGATIVE	NEGATIVE		NEGATIVE
U69-NO	NEGATIVE	NEGATIVE	NEGATIVE		NEGATIVE
U70-NO	NEGATIVE	NEGATIVE	NEGATIVE		NEGATIVE
U71-NO	NEGATIVE	NEGATIVE	NEGATIVE		NEGATIVE
U72-NO	NEGATIVE	NEGATIVE	NEGATIVE		NEGATIVE
U73-NO	NEGATIVE	NEGATIVE	NEGATIVE		NEGATIVE
U74-NO	NEGATIVE	NEGATIVE	NEGATIVE		NEGATIVE

Table 2: Results of tests performed in Novara.

Only two patients are positive for IgG in the ELISA assay (2.7%), including one who is strongly positive (U12-NO) and the other who is weakly positive (U35-NO).

Regarding IgM in immunoenzymatic assay, 6 samples are positive (8.1%), of which 2 are high positive (U28-NO and U34-NO) and 4 are low positive (U35-NO, U48-NO, U55-NO and U63-NO).

An example of the results obtained in ELISA is shown in the image. Positive samples take on a yellowish tint. U12-NO is in position D2 on the below plate. (Fig.17)

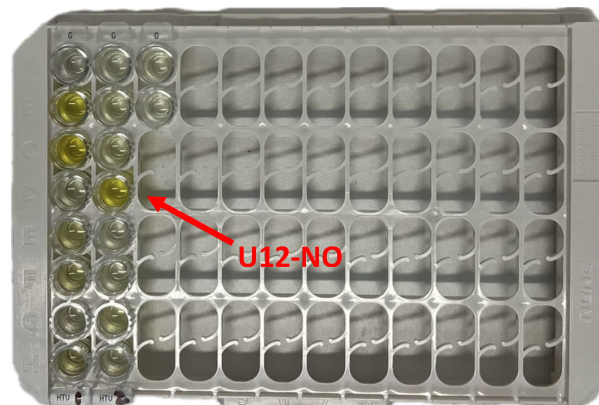

Figure 17: ELISA plate.

11 samples are positive for IgG detection by indirect immunofluorescence assay, between these 6 are weak positive. More in detail, 4 of these are positive for Saaremaa virus (U12-NO, U13-NO, U63-NO and weakly U67-NO), 2 are positive for Dobrava (U28-NO and weakly U56-NO), 1 is positive for Puumala (U31-NO), 1 is weak positive for Seoul (U64-NO), 1 low positive for Sin Nombre (U43-NO) and 2 are weak positive for the virus Hantaan (U35-NO and U41-NO). The overall positivity is 14.9%. However, considering only strong positives (5 out of 11), the prevalence is 6.8%.

In the immunofluorescence test for the IgM only 9 samples are positive. Among these, 8 are weak positive and 1 strong positive. Specifically, 3 are weak positive for Saaremaa (U36-NO, U38-NO, U41-NO), 1 is positive for Saaremaa (U65-NO), 3 are weak positive for Sin Nombre (U42-NO, U47-NO and U61-NO) and the last 2 are weak positive for Puumala (U49-NO) and Seoul (U48-NO) respectively. The prevalence of IgM is 16.1%.

Following there are some examples of IFA results. The first two photos represented a positive and a negative control. While in the next is possible to see the IgG positivity for Saaremaa (U12-NO) and for Dobrava (U63-NO). (Fig 18)

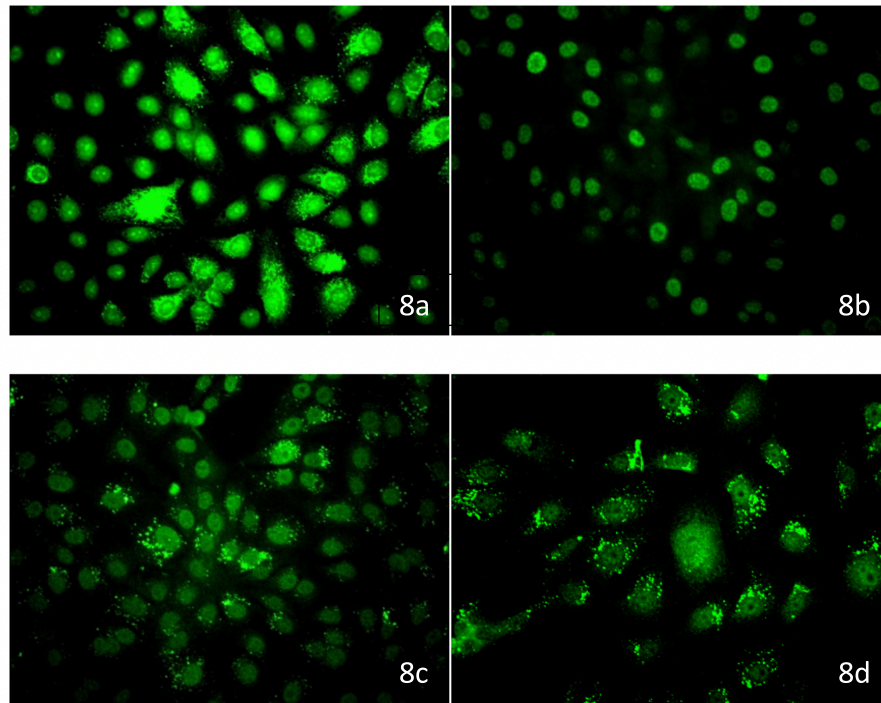

Figure 18: IFA microscope image. 8a) Ctr pos, 8b) Ctr neg, 8c) U012-NO 8d) U063-NO.

The sample U12-NO results positive for both ELISA IgG and IFA IgG (Saaremaa), while the sample U35-NO is weak positive for ELISA IgG and IgM, but also for IFA IgG (Hantaan).

	ELISA IgG	ELISA IgM	IFA IgG	IFA IgM
POS	2 (2,7%)	6 (8%)	11 (14,9%)	9 (16,1%)
NEG	72 (97,3%)	68 (92%)	63 (85,1%)	47 (83,9%)

Table 3: Results' summary table.

In general, 21 patients result positive to the different tests performed. The majority of them (57,1%) are male. 15 declared in the form to live in a house in the country (71,4%). Moreover, a low proportion during the day perform different outdoor activities in place where rodents can be present (42,9%) and finally the 38,2% are farmers or veterinarians or breeders.

CHARACTERISTICS OF THE POSITIVE SAMPLES (n.21)		
SEX	M	12 (57,1%)
	F	9 (42,9%)
WORKING CONDITION	Farmers	6 (28,6%)
	Breeders	1 (4,8%)
	Veterinarians	1 (4,8%)
LIVING CONDITION	Country House	15 (71,4%)
	Outdoor Hobbies	9 (42,9%)

Table 4: Main characteristic of the positive patients.

DISCUSSION

From the results obtained, it can be seen that the positivity rates are very high. Among the 74 patients tested so far, 21 samples are positive for one or more of the tests performed. This number is equal to 28% of the total number of subjects that were recruited, so it represents a higher percentage respect the ones reported in other European countries and would confirm an active presence and circulation of the Hantavirus in Italy. Even considering only samples with a strong positivity (7) and thus excluding weak positive samples, the overall rate is 9.45%, which is still higher than previous works in Italy. In our case, it is possible to compare because one of the studies used to assess prevalence was carried out in Novara (Faolotto et al.). In this study, the rate of positivity for Hantavirus was 7%, which is much lower than the rates we have seen in our study so far. The results coming from the other regions are not yet know, but they will be very interesting to begin to better define a geographical map of the distribution of these viruses on the Italian territory.

This noticeable percentage increase confirms a trend previously seen in Trentino-South Tyrol between the years 2006 and 2015, in which the percentage had increased from 0.2% to the 4% of the population (Kallio-Kokko et al, Tagliapietra et al). The reason for this change may be due to changed climatic conditions in recent years with seasons characterized by heavy rains alternating with dry seasons. This could encourage rodents to proliferate and spread these viruses.

Another very interesting finding is the positivity for viruses belonged to different serogroups and not only those highlighted by previous studies (DOBV and PUUV). In particular, a high percentage was found for Saaremaa virus (8 out of 21 positive cases). This virus has already been described in Europe, but never in Italy. So, our study would be the first evidence of the

presence of Saaremaa in our country. However, other viruses were also detected in our study (Seoul, Hantaan and Sin Nombre), but in this case the weak antibodies positivity may indicate cross-reactions, that would require further evaluations.

Inclusion of at-risk occupations (breeders, farmers and veterinarians) results in a 33.3% positive rate, higher respect the percentage in general population (28%). This finding would tend to confirm an increased risk of infection in individuals performing these work activities.

A very interesting case were represented by the two samples U12 and U38. The subject U12 is a 10-year-old boy whose parents reported that he had contact with a rodent a few months prior to the collection. He results strongly positive for IgG on ELISA and also for Saaremaa virus on IFA investigation. Both parents were sampled in turn. The mother (U38) is weakly positive for IgM in immunofluorescence for Saaremaa virus.

The data obtained show an important difference between IgG positivity detected by the ELISA method and the IFA method. (Table 5)

IgG	ELISA +	ELISA -
IFA +	2	9
IFA -	0	63

Table 5: ELISA positivity.

The table shows that out of 11 samples positive in IFA only 2 are positive in ELISA. This discrepancy may have several explanations: first, the ELISA test detects Ab directed against only 3 viruses, unlike the IFA test allows the detection also of IgG directed against Saaremaa, Seoul and Sin Nombre. It is precisely the high number of Saaremaa virus positives in our

case series that may be a partial reason for the lack of positivity in the ELISA. In any case, the ELISA test seems to be less sensitive than the IFA, and therefore we consider the performance of the IFA to be better for a correct diagnosis of infection.

Looking at the results for IgM detection, we see a higher prevalence of positivity than for IgG, both in IFA (16.1% vs. 14.9%), but especially in ELISA (8% vs. 2.7%). However, the discordant cases turn out to be weakly positive for IgM. This seemingly surprising discordance can be explained by the possibility of false-positive or non-specific reactions that can occur for IgM. In particular, the presence of rheumatoid factor could produce these results. We plan to investigate this phenomenon in the future by evaluating the actual presence of interferents.

In contrast, cases with a high positive signal for IgM in both IFA and ELISA (3 cases) may be more indicative of recent infections. The presence of recent infections more strongly indicates the active circulation of these viruses in our territory.

So far, this work is based on preliminary and therefore not definitive data. Certainly, in the future, when the final goals of sample collection for all 6 centers are reached, it will be easier to draw more certain conclusions. It will also be important to collect data from the other tests that have not yet been performed, in particular: Western Blot analysis for high-positive and low-positive samples, molecular studies for viral genome detection by RT-PCR in positive cases, and the performance of metagenomic tests on samples positive in molecular tests. In addition, neutralization testes could be very useful to reduce false-positive results caused by cross-reactivity for both IgM and IgG antibodies.

The results of investigations carried out on rodents and bats by the two zooprophylactic institutes participating in the study will also be of great interest. Veterinary analysis could

also support the data obtained in humans and define the distribution and circulation of Hantavirus in our territory.

In conclusion, although these data are still preliminary, we can assume that Hantaviruses are present in Italy with a high degree of active circulation and are increasing compared to the past. It also seems that some professions (farmers, breeders and veterinarians) are more at risk than others. Based on these data, it is important to involve clinicians in the future. They can pay more attention to the possible pathologies caused by these infections in order to improve the identification of these forms and their possible treatment.
For proper diagnosis, IFA seems to be the most reliable test. We believe that all level II laboratories in Italy should be equipped with diagnostics to detect Hantavirus infections in a timely manner.

We also believe that it is important to have a continuous epidemiological surveillance, both at human and veterinary level, in order to identify the most endangered areas in the whole territory, including regions that have not been considered yet in this project.

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