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Genetic characteristics of field and attenuated rabies viruses and molecular epidemiology of rabies in Finland and Russia

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ACADEMIC DISSERTATION

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Content

List of abbreviations

ABLV – Australian bat Lyssavirus

BHK-21 – baby hamster kidney cell culture

CCIT – cell culture inoculation test

CNS – central nervous system

CVS – challenge/control virus strain

cDNA – complementary DNA synthesized from RNA template

DEAE-dextran – diethylaminoethyl-dextran

EBLV – European Bat Lyssavirus

ELISA – enzyme-linked immuno assay

ERA – Evelyn Rokitniki Abelseth strain

FAT – fluorescent antibody test

FAVN – fluorescent antibody virus neutralization test

FITC-globulin – fluorescein isothiocyanate labeled globulin

IU – international unit

IHCT – immunohistochemical test

LAT – latex agglutination test

mAb – monoclonal antibody

MEM – Eagle's minimal essential medium

MIT – mouse inoculation test

MNA – murine neuroblastoma cell culture

PV – Pasteur Virus strain

PM – Pitman-Moore strain

RT-PCR – reverse transcriptase polymerase chain reaction

RNP – ribonucleoprotein

SAD – Street-Alabama-Dufferin strain

TCID – tissue culture infectious dose

VNA – virus neutralizing antibody

Abstract

Rabies is a fatal disease that affects the central nervous system of all warm-blooded mammals. The rabies virus belongs to the order *Mononegavirales*, family *Rhabdoviridae*, genus *Lyssavirus*. This virus has a negative single-stranded RNA genome and the virions are bullet-shaped.

Rabies is reported in many countries throughout the world and has been registered in all continents except Australia, where only the bat Lyssaviruses have been found, and in Antarctica where the main vectors of rabies are absent. Russia and most of the bordering countries are affected by rabies. Finland was a rabies-free country from 1959 to 1988, when a sylvatic rabies epidemic appeared with raccoon dogs as the main host and vector of infection. That epidemic was eradicated by the oral vaccination of wild carnivores and the parenteral immunization of dogs and cats; and Finland has been rabies-free since 1991. However, this status is constantly under threat because rabies is endemic in Russia and Estonia. In June 2003, a horse imported to Finland from Estonia was clinically and laboratory diagnosed as rabies positive. The close relationship of the isolated equine virus strain with the current Estonian strains was verified during subsequent molecular epidemiological studies. Because the case was imported, it did not affect Finland's rabies-free status. Also in 2007 another 2 imported cases of rabies were recorded: one in a human being from Philippines and the other in a dog from India.

Five different antigenic variants of the rabies virus were identified among rabies positive field samples from Russia, Finland, and Estonia by using antinucleocapsid monoclonal antibodies. Two rabies virus field isolates showed a different reaction pattern that was similar to that of the vaccine strains of the SAD group, which might suggest a new antigen variant or reverted vaccine strain. Nevertheless, the sequence analysis showed that the vaccine strains RV-97 and SAD B19 included in the oral anti-rabies vaccine "Sinrab" (Russia) and "Fuchsoral" (Germany), respectively, differ considerably from all the field strains.

Field rabies viruses collected in recent years from different regions of the Russian Federation were chosen on the basis of mAb studies and geographical origin for molecular epidemiological studies to characterize their genetic heterogeneity and to study their molecular epidemiology. In addition to the Russian viruses, archival samples from Estonia and Finland and Russian vaccine strains were also included in this study. Among the field viruses studied, two main phylogenetic groups were found, and designated as the Pan-Eurasian and Caucasian based on their geographical origin. The Pan-Eurasian

group including some reference viruses from Europe was further divided into four subgroups. All the vaccine strains were clearly different from the field strains. No recombination between the field and vaccine virus strains was observed. The critical roles of geographical isolation, the limitation of the genetic clustering, and the evolution of the rabies virus were shown during this study.

The rabies virus vaccine strain RV-97 is widely used in Russia as a component of the oral anti-rabies vaccine “Sinrab”. To characterize the molecular properties of this strain, entire genome sequencing was conducted. A simple technique was developed to obtain this sequence, including the 3’- and 5’- ends. The entire genome sequence and deduced amino-acid sequences of the major viral proteins were compared with the sequences of other known fixed rabies viruses. The strain RV-97 formed a separate phylogenetic branch and seems to be more related to the group of Japanese strains. The field strains from the Caucasian group seem to be phylogenetically the nearest group to the RV-97 strain.

The data shown herein makes it possible to develop molecular methods for distinguishing between the field rabies viruses from the vaccine strains for the rapid recognition of the vaccine strains that are unstable or have reverted back to their pathogenic form. The wide genetic heterogeneity verified in this study indicates that it is important to remain on permanent alert for the appearance of rabies.

List of original publications

I. Metlin A.E., Cox J., Rybakov S.S., Huovilainen A., Grouzdev K., Neuvonen E., 2004. Monoclonal antibody characterization of rabies virus isolates from Russia, Finland and Estonia. Journal of Veterinary Medicine Series B 51:94-96.

II. Metlin A.E., Rybakov S.S., Gruzdev K.N., Neuvonen E., Cox J., Huovilainen A., 2006. Antigenic and molecular characterization of field and vaccine rabies virus strains in the Russian Federation. Developments in Biologicals 125:33-37.

III. Metlin A.E., Holopainen R., Tuura S., Ek-Kommonen C., Huovilainen A., 2006. Imported case of equine rabies in Finland: clinical course of the disease and the antigenic and genetic characterization of the virus. Journal of Equine Veterinary Science 26:584-587.

IV. Metlin A., Rybakov S., Gruzdev K., Neuvonen E., Huovilainen A., 2007. Genetic heterogeneity of Russian, Estonian and Finnish field rabies viruses. Archives of Virology 152:1645-1654.

V. Metlin A., Paulin L., Suomalainen S., Neuvonen E., Rybakov S., 2007. Mikhalishin V., Huovilainen A. Characterization of Russian rabies virus vaccine strain RV-97. Virus Research, doi:10.1016/j.virusres.2007.11.016

1. Literature review

1.1. Definition and history of rabies

Rabies is a fatal viral zoonosis which causes encephalitis in many warm-blooded animals and humans. There have been indications relative to the occurrence of rabies since the time of Homer (eighth century B.C.) onwards. The first archival appearance of the disease was in the fourth century B.C., but precise diagnosis was not possible before the first century B.C. (Blancou, 1994, 2004; Neville, 2004). The first human rabies vaccine was developed in 1885 by Louis Pasteur and since then, significant developments have been made in this field including progress in laboratory diagnosis, vaccination and rabies control in wild, domestic and farm animals (King et al., 2004).

1.2. Rabies virus characteristics

1.2.1. Classification

The rabies virus belongs to the order *Mononegavirales*, family *Rhabdoviridae*, which includes at least three genera: *Lyssavirus, Ephemerovirus,* and *Vesiculovirus* (Virus Taxonomy, 2005). The *Lyssavirus* genus is further divided into seven genotypes. The genotype 1 is known to be the most widespread and comprises the classical field rabies viruses, and the laboratory and vaccine strains. The rabies-related viruses isolated in the African continent belong to genotypes 2, 3, and 4, with prototypes of the Lagos bat virus, Mokola virus, and Duvenhage virus, respectively. The viruses isolated from bats in Europe represent genotypes 5 and 6 (EBLV1 and EBLV2). The Australian bat Lyssavirus (ABLV) represents the seventh genotype (Gould et al., 1998; Guyatt et al., 2003).

It has been found that some new rabies virus genotypes exist among viruses isolated from the territory of the former Soviet Union. It was proposed that the Aravan virus isolated from a bat in Kyrgyzstan should be classified as the eighth genotype (Arai et al., 1997). The Khujand virus isolated in Tajikistan can also be classified as a separate genotype of Lyssavirus (Kuzmin et al., 2003). The existence of two additional Lyssavirus genotypes among strains isolated from bats (Irkut virus and West Caucasian Bat Virus) in the Russian Federation was also recently proposed (Botvinkin et al., 2003; Kuzmin et al., 2005).

1.2.2. Structure of virion, genome, and proteins of rabies virus

The virions of the rabies virus have a bullet-shaped structure (Fig. 1) with a diameter of 75 nm and length of 100-300 nm (Matsumoto 1962; Tordo and Poch, 1988). The virion contains two major subunits. The first subunit is the ribonucleoprotein (RNP) of cylindrical and compact form located in the central part of the virion, the second subunit is a thin surrounding envelope covered with spiky projections (Tordo, 1996).

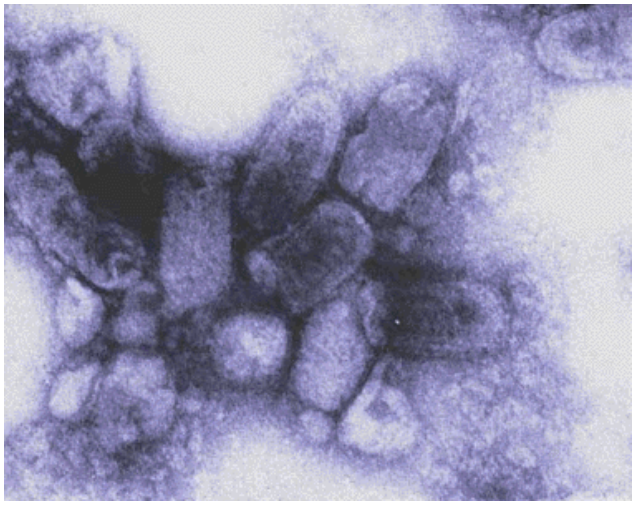

Figure 1. Rabies virus virions (http://www.wadsworth.org/databank/rabies.html).

The genome of the rabies virus is a non-segmented negative-sense single-stranded RNA of approximately 12000 nucleotides (nt) in length (Fig. 2). The virus RNA codes 5 major proteins: the nucleoprotein (N protein), phosphoprotein (P protein), matrix protein (M protein), glycoprotein (G protein), and the RNA-dependent RNA-polymerase (L protein). The RNP complex is formed by the N, P, and L proteins associated with the viral RNA. The RNP is surrounded by a lipid bilayer associated with the G and M proteins (Goto et al., 2000). The genome also contains several non-coding regions including a G-L intergenic region called the “pseudogene” or Ψ-region (Fig. 2), which became suppressed and degenerated during the evolution of the virus (Tordo et al., 1986).

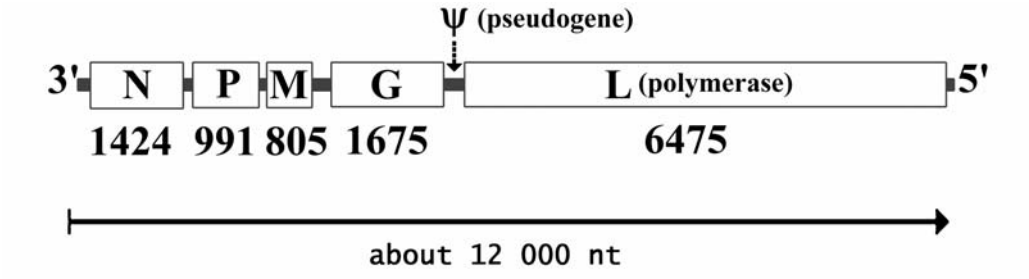

Figure 2. Schematic structure of the rabies virus genome (adapted from http://www.cdc.gov/ncidod/dvrd/rabies/the_virus/virus.htm).

The nucleoprotein consists of 450 amino acids and participates in the transition from RNA transcription to replication. It encapsidates the positive-strand leader RNA and prevents further transcription of the genomic RNA (Wunner, 1991; Yang et al., 1998) and contains several antigenic and immunodominant sites. The antigenic sites I and III include stretches of amino acids at positions 374-383 and 313-337, respectively (Tordo, 1996). One of the immunodominant sites is known to be located at position 404-418 (Dietzschold et al., 1987; Ertl et al., 1989). Recently, the crystal structure of the rabies virus nucleoprotein-RNA complex was determined (Albertini et al., 2006).

The P protein is a phosphorylated protein of 297 amino acids, associated with the L protein to function as a noncatalytic cofactor for the RNA polymerization, and with the N protein to support adequate RNA encapsidation (Chenik et al., 1994, 1998; Jacob et al., 2001). Four additional proteins derived from the phosphoprotein gene of the rabies virus were also found to be present in infected cells, cells transfected with a plasmid encoding the wild-type P protein, and in the purified virus. It was shown that these proteins are initiated from secondary downstream in-frame AUG initiation codons. The P-gene is the only gene shown to encode more than one protein (Chenic et al., 1995). Two antigenic sites were found to be located at positions 75-90 (Tordo, 1996). The domain 83-172 was shown to contain the major antigenic determinants (Raux et al., 1997). An immunodominant site was also mapped in phosphoprotein at positions 191-206 (Larson et al., 1991), and was identified as the responsible alpha/beta interferon antagonist (Brozka et al., 2005).

The matrix protein is a 202 amino acid polypeptide which plays a key role in virus assembly and binding. It covers the ribonucleoprotein (RNP) coil to keep it in a condensed form and was found to interact specifically with the glycoprotein (Mebatsion et al., 1999). The major antigenic site is located between the amino acid residues 1 and 72 (Hiramatsu et al., 1992).

The glycoprotein is a 524 amino acid protein and is the most studied protein of the rabies virus. It has a trimeric structure (Gaudin et al., 1992), with the first 19 amino acids representing the signal domain which is found only in nascent protein. The glycoprotein contains several antigenic sites and epitopes. The antigenic epitope I is represented by a single amino acid at position 231 of the mature glycoprotein. The site II is known to be discontinuous and involves two separate stretches between positions 34-42 and 198-200. The antigenic site III is located at position 330-338, epitope VI at position 264, “a” – 342-343 (Lafon et al., 1983, 1984; Seif et al., 1985; Prehaud et al., 1988; Bunschoten et

al., 1989; Benmansour et al., 1991).

The major immunodominant site of the glycoprotein is located between amino acids 222 and 332 (Johnson et al., 2002b). The amino acid at position 333 of the mature glycoprotein was found to be associated with viral pathogenicity (Seif et al., 1985; Badrane et al., 2001). It was shown that either arginine or lysine at position 333 of the ERA and CVS fixed rabies virus strains is necessary for rabies virulence in adult mice (Tuffereau et al., 1989).

The L protein is the largest protein of the rabies virus and L gene occupies more than half of the virus genome. It has been the least studied rabies protein at biochemical and immunological levels, but the best analyzed theoretically (Tordo, 1996). The L-protein is associated with the RNP, RNA-dependent RNA polymerase. It participates in transcription by making five individual mRNAs, one for each viral protein.

1.3. Virus-host interaction

1.3.1. Pathogenesis

Rabies is a central nervous system (CNS) disease that is almost invariably fatal (Dietzschold et al., 2005), except for few rare reported cases (Miah et al., 2005). The site of rabies virus entry into the host is usually at the skin or mucosal membrane where the virus is introduced into the deeper layers of the skin or into the muscular tissue through biting, licking, or scratching by a rabies-infected mammal, usually a carnivore or a bat (Charlton, 1994). The transmission of rabies can also occur under unusual circumstances: by inhalation of large amounts of aerosolized rabies virus, and through organ transplantation from rabies infected patients (Gode and Bhide, 1988; Krebs et al., 1995; Hellenbrand et al., 2005; Smith et al., 2006). Rabies-infected animals have in their salivary glands high titers of the rabies virus, which can be even greater than in the brain (Charey and McLean, 1983). There are marked differences between the different strains of virus and their ability to infect, spread within the body, and produce disease (Kaplan, 1996; Dietzschold et al., 2005). It has been suggested that the attenuated rabies viruses activate the host's innate immune and antiviral responses, while these responses are evaded by the pathogenic rabies viruses (Wang et al., 2005). The course of the disease can be different between animal species: for example, foxes have a comparatively shorter morbility period than skunks (Sikes, 1962).

After the bite (Fig. 3), the virus particles "travel" to the nearby nerves and then along the nerve fibers to the brain at a speed of a few millimeters per day (Jackson,

1991). It was suggested that the virus is propagated from the entry point to the CNS due to the interaction between the P protein of the rabies virus and the dynein light chain LC8 (Poisson et al., 2001).

A bite on the head or neck will usually cause symptoms more quickly than a bite on the hind leg. However, when the virus has entered the nerve endings, it advances relentlessly up the nerve bodies until it reaches the spinal cord and eventually the brain. From the brain, the virus can spread to other tissues - the salivary glands, respiratory system, and the digestive tract (Krebs et al., 1995).

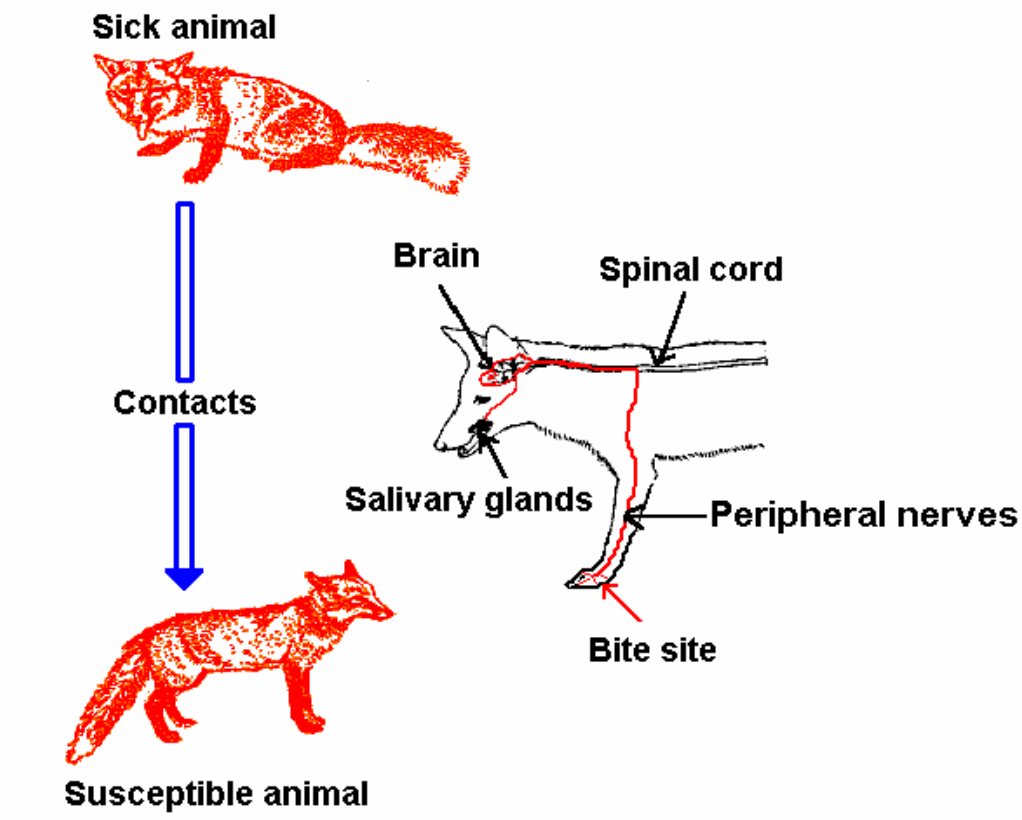

Figure 3. Spread of the rabies virus from the bite site to the CNS (by Bacon & Macdonald, 1980).

1.3.2. Clinical signs

The clinical signs of rabies are known since the ancient times (Blancou, 1994). The duration from bite to the appearance of clinical signs varies significantly, ranging from a few days to a several months. The clinical signs may appear only after the involvement of numerous neurons, and death may occur as a result from the involvement of vital nerve centers (Schneider, 1975). There are three phases described in the clinical course of rabies:

Prodromal period – the first 1 to 3 days after the rabies virus reaches the brain. Vague neurological signs that progress rapidly - some animals may appear tamer, some will demonstrate intense drooling. Death usually follows within 10 days, due to paralysis.

Excitative stage – the next 2 to 3 days. This is the "furious rabies" stage - tame animals suddenly become vicious, attacking humans and other animals as they roam and wander. Some animals will chew and eat odd objects (rocks, sticks, etc.). Paralysis then begins, and loss of the ability to swallow will cause frothing at the mouth of the affected animal.

Paralytic stage - follows the excitative stage, or is the main clinical presentation for some animals. The throat and chewing muscles are paralyzed, and the animal is unable to swallow, causing excessive drool, the lower jaw is often dropped. This is a dangerous period for human contact with domestic animals such as cows and horses; "choke" (foreign body within the throat) can be a misdiagnosis of rabies, causing humans to be potentially exposed as they investigate the problem. Similar situations occur in dogs that appear to be choking (drooling and dropped jaw). This is also the period when wild animals may seem tame to humans and nocturnal animals are seen in the daylight. The paralysis progresses from the neck and jaw to all areas of the body, the animal falls into a coma, and dies within a few hours.

In bats, the clinical signs of a Lyssavirus-infection include loss of body mass, lack of coordination, muscular spasms, agitation, increased vocalization, and overt aggression (Brass, 1994; Whitby et al., 2000; Shankar et al., 2004), but in many cases, rabies in bats can be clinically silent and left unnoticed before dead animals are found and laboratory tests are performed (Ronsholt et al., 1998). When bats were found alive, the clinical signs were generally described as paralysis, unprovoked vocalization, and aggression (biting) during handling (Rabies bulletin Europe, 1989). However, almost all bats will bite when handled (Vos et al., 2007).

Because dogs are often responsible for the transmission of rabies to humans, clinical signs in this species are more elaborately described, studied, understood, and include: drooping jaw, abnormal sounds when barking, dry drooping tongue, licking of it’s

own urine, abnormal liking of water, regurgitation, altered behavior, the biting and eating of abnormal objects, aggression, biting without provocation, running without no apparent reason, stiffness upon running or walking, restlessness, biting during quarantine, sleepy appearance, gait imbalance, and frequent demonstration of the “Dog sitting” position (Tepsumethanon et al., 2005).

1.3.3. Pathological changes

The pathology of rabies infection is typically characterized by encephalitis and myelitis. When brain tissue from rabies virus-infected animals is observed with a histological stain, such as hematoxylin-and-eosin, evidence of encephalomyelitis may be recognized by light microscopy (Fig. 4 A&B). A perivascular non-suppurative infiltration is the most frequently noted histological alteration in rabies (Perl, 1975). The cytoplasmic eosinophilic inclusion bodies (Negri bodies) can often be found in rabies-infected neurons (Fig. 4 C&D), especially in the pyramidal cells of the hippocampus and the Purkinje cells of the cerebellum (Negri, 1903). These Negri bodies may vary in size from 0.25 to 27 μm, and these inclusions have been defined as areas of active viral replication by the identification of rabies viral antigen (Schneider et al., 1975).

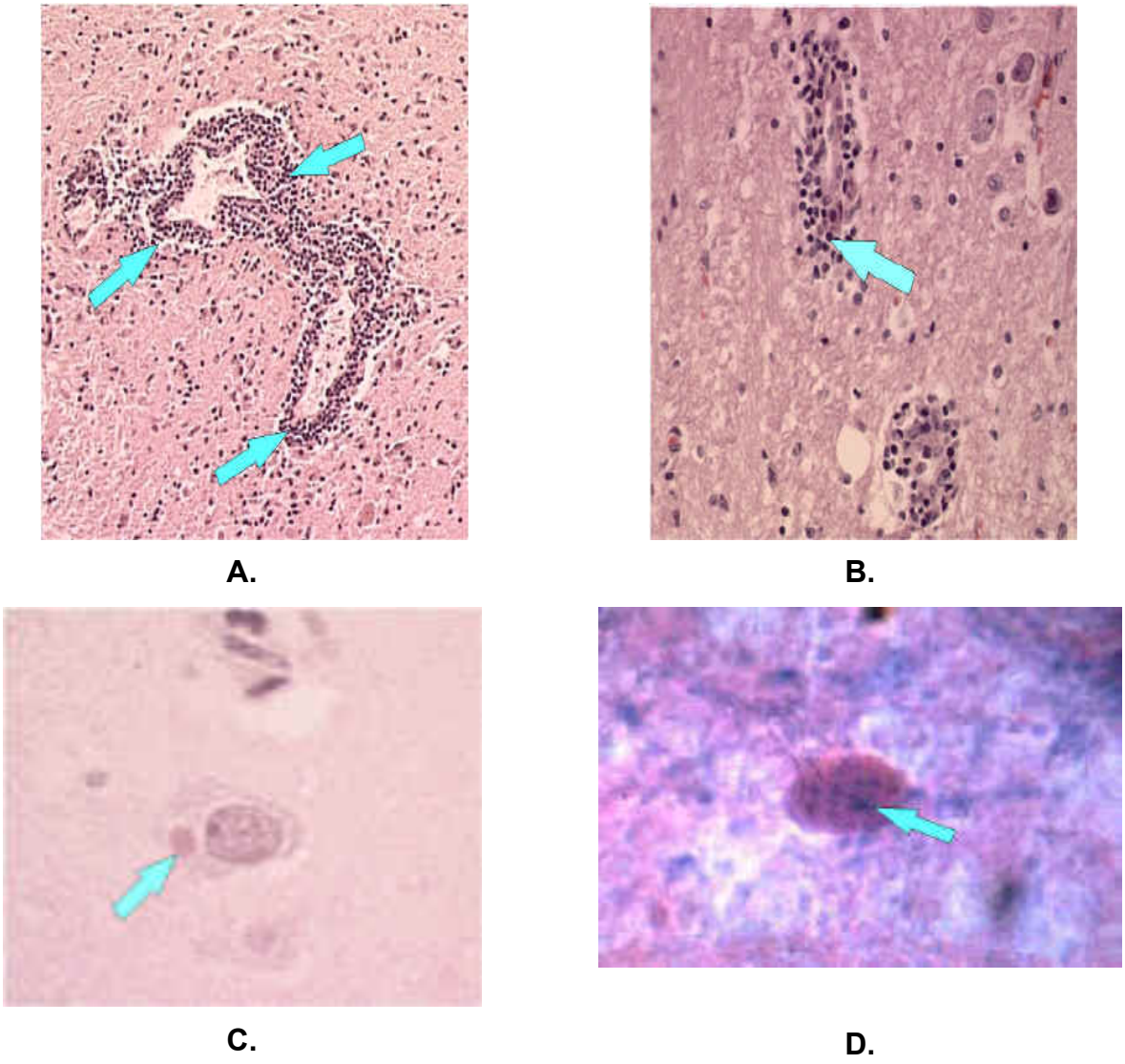

Figure 4. A&B. Perivascular cuffing or inflammation around a blood vessel. Perivascular inflammatory cell infiltrates in hematoxylin-and-eosin stained brain tissue. **C&D.** Negri bodies stained with hematoxylin and eosin (**C**) and Sellers stain (**D**). All pictures were taken from http://www.cdc.gov/ncidod/dvrd/rabies/diagnosis/images.

1.4. Laboratory diagnosis of rabies

The diagnosis of rabies has to be quick and reliable in order to evaluate the risk of infection to the exposed individual (Zimmer et al., 1990), and it is also important for health authorities responsible for the surveillance and control of the epidemics and epizootics (Perrin et al., 1986). The techniques for the diagnosis of rabies are standardized internationally, and several tests are available presently. The detection of Negri bodies in brain smears and the histological examination were the first methods for diagnosing rabies, but these are not currently used widely because of their low sensitivity and due to the availability of highly sensitive and specific modern techniques which can be subdivided into three main groups:

- ***Detection of viral antigen*** – fluorescent antibody test (FAT), enzyme-linked immunoassay (ELISA), immunohistochemical test (IHCT), and latex agglutination test (LAT);
- ***Detection and identification of viral genome*** – reverse transcriptase polymerase chain reaction (RT-PCR) with subsequent nucleotide sequencing;
- ***Virus isolation*** – mouse inoculation test (MIT) and cell culture inoculation test (CCIT).

The above mentioned methods will be discussed in the specific subchapters below.

1.4.1. Detection of viral antigen

Fluorescent antibody test. In 1958, Goldwasser and Kissling reported that the fluorescent antibody technique could be used to demonstrate rabies virus antigens in brain tissues of experimentally infected mice. Further studies have shown this method to be an efficient tool for the diagnosis of rabies, and it later became the reference method for the diagnosis of this infection (Beauregard et al., 1965). This technique implies the preparation of smears, impressions or cryosections from brain tissues (Ammon's horn, cerebellum, cerebral cortex, and the brain stem), tissue fixation, mostly in cold acetone, and staining with fluorescein isothiocyanate-labeled polyclonal or monoclonal anti-rabies antibodies (Kissling, 1975; Dean et al., 1996; OIE, 2004). The FAT allows specific and highly sensitive detection of the rabies virus antigens in brain smears, salivary gland sections, and infected cell cultures. It can be used for the intravitam rabies diagnosis in

the skin biopsies (Bryceson et al., 1975; Warrell et al., 1988) and to stain rabies virus antigens in the salivary glands (Goldwasser et al., 1959).

It is also possible to perform the FAT with formalin-fixed paraffin-embedded brain sections using digestion with proteases, such as pepsin or trypsin (Johnson et al., 1980; Umoh and Blenden, 1981; Barnard and Voges, 1982; Reid et al., 1983; Metlin et al., 2007).

Enzyme-linked immunoassay test is a rapid technique that facilitates the evaluation of a large number of samples simultaneously, which is performed on microplates previously sensitized with anti-rabies immunoglobulin. Suspensions of homogenized material are placed on the wells of microplate for specific binding which can be revealed by the use of a peroxidase conjugate (Perrin et al., 1986). Additionally, a quantitative ELISA (N-ELISA) for rabies virus detection based on the quantitation of nucleoprotein (N) in rabies virions captured by rabies-virus-specific polyclonal antibodies on an ELISA plate can be used for the quantitative detection of both infective and defective interfering particles of rabies virus (Katayama et al., 1999).

Immunohistochemical testing is predominantly used for research purposes and allows the perfect identification and localization of rabies virus antigens, and is ideal for retrospective diagnosis (Johnson et al., 1980; Fekadu et al., 1982; Torres-Anjel et al., 1984; Palmer et al., 1985; Shin et al., 2004). This method is usually used to stain histological paraffin sections after deparaffinization, rehydration, and digestion with a proteolytic enzyme (proteinase K etc.). For the specific staining of rabies virus antigen by immunohistochemical testing, the primary anti-rabies serum, anti-species serum, and the peroxidase complex are used (Bourgon and Charlton, 1987; Sinchaisri et al., 1992).

Latex agglutination test (LAT) is a simple and rapid technique, which may be used more widely in the laboratory diagnosis of rabies in the future. It has been used to detect rabies virus antigens in the saliva of dogs with 99% specificity and 95% sensitivity. The essence of the LAT is inducing agglutination on a glass slide using polystyrene latex beads coated with anti rabies IgG (Kasempimolporn et al., 2000, 2007).

1.4.2. Detection of viral genome

The reverse transcriptase polymerase chain reaction (RT-PCR) with subsequent nucleotide sequencing permits the diagnosis of rabies, typing, and molecular epidemiological studies. Since the rabies genome is RNA, the amplification procedure consists of the reverse transcription of the target RNA strain into complimentary DNA

(cDNA), followed by the amplification of the cDNA by PCR (Tordo et al., 1995, 1996).

The RT-PCR procedure consists of the following steps: total RNA extraction, cDNA synthesis with random or specific primers, amplification of the cDNA with specific primers, and visualization of the results with horizontal electrophoresis in agarose gel containing ethidium bromide observed under UV light (Heaton et al., 1999).

The RT-PCR is widely used for rabies diagnosis, and different parts of the genome can be targeted for this reason, but in most cases the N gene is utilized (Kulonen and Boldina, 1993; Bourhy et al., 1999; Ito et al., 2003; Losa-Rubio et al., 2005). A rapid RT-PCR technique was developed for the detection of the classical rabies virus (genotype 1) and the rabies related EBLVs (genotypes 5 and 6), and also to distinguish between the six established rabies and rabies-related virus genotypes (Black et al., 2000, 2002). The PCR can also be applied to detect the rabies virus genome in formalin-fixed paraffin-embedded brain tissue (Kulonen et al., 1999) and for the intravitam diagnosis of rabies in humans by testing saliva and cerebrospinal fluid (Crepin et al., 1998). The Real-time PCR is a quantitative technique which allows the detection of an increase in the amount of DNA (cDNA) during amplification. It is currently used for the ante- and post-mortem diagnosis of rabies and the discrimination of the Lyssavirus genotypes (Wakeley et al., 2005; Nagaraj et al., 2006; Saengseesom et al., 2007).

1.4.3. Virus isolation

The mouse inoculation test (MIT) was one of the first diagnostic tests for rabies. Laboratory mice are inoculated intracerebrally or subcutaneously with the supernatant of the sample suspension. The inoculated mice must be observed for up to 30 days after inoculation. Death during the first 48 hours after inoculation must be considered as non-specific; all the animals dead after this period must be dissected and brain samples must be tested for rabies by the FAT (Koprowski, 1996). This method can be used for testing the brain and salivary gland suspensions, as well as the saliva samples, for the presence of live rabies virus (Adeiga and Audu, 1996; Delpietro et al., 2001).

The cell culture inoculation test has already replaced the MIT in many countries and implies the isolation of rabies virus in a cell culture monolayer with visualization by the FAT. A number of cell lines have been selected and tested: cow brain cells (CB3), cerebral and cerebellar grey matter cells of mice (MBC-2, MBC-3), chicken embryo fibroblasts (CEF), murine, feline and human glial cultures, human monocytic U937 and THP-1 cells, murine macrophage IC-21, murine monocytic WEHI-3BD- and PU5-1R

cells, murine monocytic P388D1 and J774A.1 cells, kidney epithelial cells derived from African green monkey (Vero), and McCoy cells (Smith et al., 1978; Clark, 1980; Celer et al., 1991; Ray et al., 1995, 1997; Nogueira, 2004). Tollis et al., (1988) compared the sensitivity of the murine neuroblastoma (MNA), baby hamster kidney (BHK-21), and the canine fibrosarcoma A-72 cells, and confirmed that the MNA cells are the most sensitive to infection with the wild strains of the rabies virus. MNA cells are currently widely used for field virus isolation and a method employing this cell culture is recommended by the WHO and the OIE (Webster et al., 1996, OIE, 2004).

1.4.4. Detection of virus neutralizing antibodies

The detection of anti-rabies virus neutralizing antibodies (VNA) is widely used to evaluate the potency of anti-rabies vaccines because the minimal level of the VNA needed to protect animals against rabies has been determined as ≥ 0.5 IU/ml (OIE, 2004).

Virus neutralization assay has also been found useful for the monitoring of Lyssaviruses among bats (Arguin et al., 2002; Lumlertdacha et al., 2005).

Virus neutralization in mice or cell cultures. The determination of the VNA was previously conducted by virus neutralization in mice (Atanasiu, 1973) and subsequently replaced by the fluorescent antibody virus neutralization (FAVN) or rapid fluorescent focus inhibition testing (RFFIT) (Thomas, 1975; Zalan et al., 1979; Smith et al., 1996; Cliquet et al., 1998). This method enables the determination of antibody levels by the neutralization of a known dose of the rabies virus (commonly the CVS strain). Serum samples are tested and compared with the neutralization of reference standard serum with an antibody level of 0.5 or 1.0 IU/ml. This test can be conducted on microplates and the results viewed with fluorescence microscopy. The registration of the results can be automated by various means: by using an inverted fluorescence microscope coupled with a video camera and color image analysis software computer system (Peharpre et al., 1999); a peroxidase conjugate can be used instead of the fluorescent conjugate, and in this case an automatic multi-channel spectrophotometer can be used for the registration of the results (Hostnik, 2000a); flow cytometry method for calculating anti-rabies VNA has also automated results registration (Bordignon et al., 2002).

ELISA can also be used to determine antibody levels in serum samples (Kasempimolporn et al., 2007).

This method can be used for assessing the efficacy of oral fox vaccination campaigns and it was demonstrated that by using commercial ready-to-use microplates sensitized with rabies virus glycoprotein, a simple and rapid ELISA technique enables the obtaining of a rabies antibody quantization in field fox serum samples (Mebatsion et al., 1989; Esterhuysen et al., 1995; Cliquet et al., 2000, 2003, 2004, 2007; Servat et al., 2007).

The latex agglutination test is also used to detect rabies-specific antibodies. Latex beads are sensitized by coating them with purified rabies glycoprotein to detect anti-glycoprotein antibodies in serum samples. The visible agglutination is observed in the positive sera with a titer ≥ 2 IU/ml within 3–5 min after mixing, while serum samples containing less than 2 IU/ml do not agglutinate (Madhusudana and Saraswati, 2003).

1.5. Rabies epidemiology

1.5.1. Rabies situation world-wide

Rabies is widely distributed throughout the world and is present in all continents except Australia, where only bat Lyssavirus has been found, and in Antarctica. Worldwide, it has been estimated that approximately 55000 persons die annually due to rabies; 99% of human rabies deaths have occurred in the developing countries. The total global expenditure on rabies prevention is well over US$ 1 billion annually (Warrel et al., 1995; WHO Expert Consultation on Rabies, 2005).

Different animal species can be responsible for viral circulation and rabies transmission in different continents and countries. Canids have been determined to be the main hosts of the rabies virus in Africa; in most cases they are also responsible for the transmission of the virus to humans. In addition to canids (domestic and wild dogs, jackals, and wolves), mongooses, and bats are involved in rabies epidemics, as occurs in Africa (Adeiga et al., 1996; Bingham, 2005). Dogs are also the primary reservoir for rabies in Thailand (Tepsumethanon et al., 2005). In the USA, several species are involved in rabies epidemics but the main reservoirs are raccoons and skunks (Krebs et al., 2003). An epizootic of raccoon rabies, begun in the USA in the late 1970s, and developed into one of the largest and most extensive in the history of wildlife rabies (Childs et al., 2000). Rabies has been detected in rodents and lagomorphs, mostly in woodchucks (Childs et al., 1997) and also in arctic foxes (Ballard et al., 2001). In addition, bats are sometimes responsible for the transmission of rabies to humans (Miah, 2005).

1.5.2. Rabies situation in Europe and in Russia

Foxes and raccoon dogs are considered to be the most susceptible species in Europe (Artois, 1992; Kihm et al., 1992; Gylys et al., 1998). According to rabies data for the years 2005 and 2006, there were 13 rabies-free countries in Europe: Belgium, the Czech Republic, Cyprus, Finland, Greece, Iceland, Italy, Ireland, Luxembourg, Norway, Portugal, Sweden, Switzerland, and Lichtenstein. With 9,830 cases of rabies (including 9 human and 35 bat cases) in 2005, the total number of cases of rabies in Europe (European countries and European parts of Russia) has increased by 80% when compared with 2004 to approximately similar level as that of 2003. This increase is based on a 2 to 2.3-fold higher reporting of rabies cases from Russia and Ukraine (Rabies Bulletin Europe, 2005). A similar tendency was observed in Turkey, where dog-mediated rabies is the main problem (Rabies Bulletin Europe, 2005; Johnson et al., 2006; Kilic et al., 2006). Finland is a rabies-free country but three imported rabies cases were recorded recently. In 2003 rabies was found in a horse imported from Estonia (Metlin et al., 2006); in 2007a human case was recorded in a Philippine male working on a cruise ship (Kallio-Kokko H., personal communication); later in the same year, another rabies case occurred in a pappy imported from India (communication of the Ministry of Agriculture and Forestry of Finland).

Wild animals, manly red fox, are still the main rabies hosts in Europe. In 2006, 9172 rabies cases were reported in Europe, 6152 of these occurred in wild animals, 2984 in domestic animals, and only 34 in bats. In addition, 2 human rabies cases were reported (Rabies Bulletin Europe, 2006).

Rabies is endemic in the Baltic countries; in 2006, 114 animal rabies cases were recorded in Estonia, 472 in Latvia, and 2232 cases in Lithuania. The number of rabies cases in animals from Estonia decreased in 2006 by 57% when compared with 2005. This may be due to the oral wildlife vaccination started in Estonia during 2005 (Niin et al., 2007). In Latvia and Lithuania, an increase in the number of animal rabies cases was recorded in 2006 compared with 2005 (an increase of about 10% in Latvia and approximately 26% in Lithuania (Rabies Bulletin Europe, 2006).

Rabies is a very serious veterinary and public health problem in the Russian Federation. The disease is widespread throughout the country (Fig. 5) and affects different animal species: farm animals (mainly cattle, pigs, sheep, goats, and horses), domestic pet animals (predominantly dogs and cats), and wild animals (red foxes,

raccoon dogs, badgers, wolves, lynx, etc.). The disease is enzootic in Russia and there is an increasing tendency in the number of rabies affected territories (Dudnikov, 2003b); also almost 10 human rabies cases are reported annually.

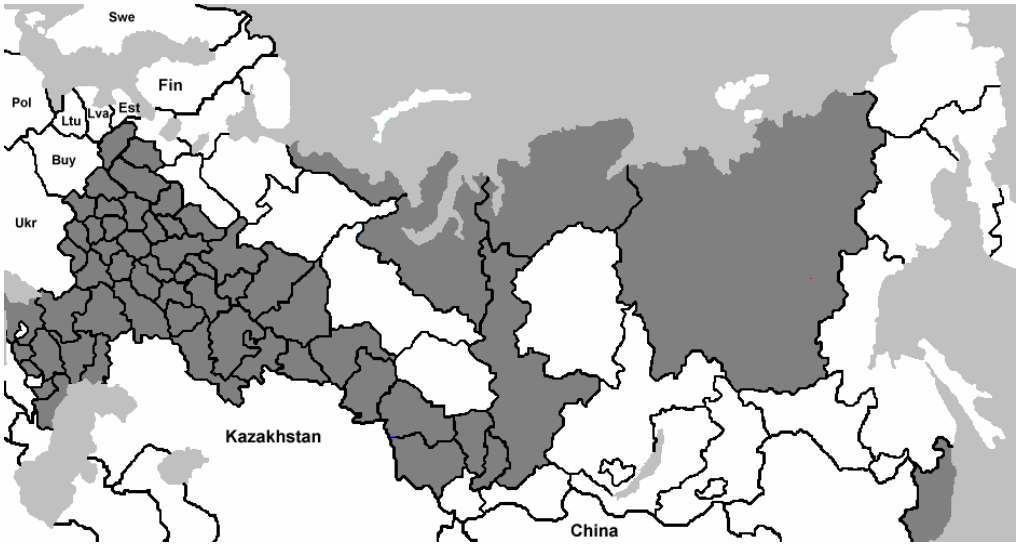

Figure 5. Map of the Russian Federation and bordering countries. The Russian regions affected by rabies (according to data recorded in 2006) are colored dark grey.

The largest outbreaks of rabies have occurred in the south-western regions of the Russian Federation and rabies is spreading further to the northern and eastern directions (Makarov and Vorob'ev, 2004). The number of animal rabies cases increased annually from 2000 to 2005, except for 2004, when a decrease in rabies cases was recorded (Fig. 6). In 2006, the number of animal rabies cases decreased substantially (approximately 57%).

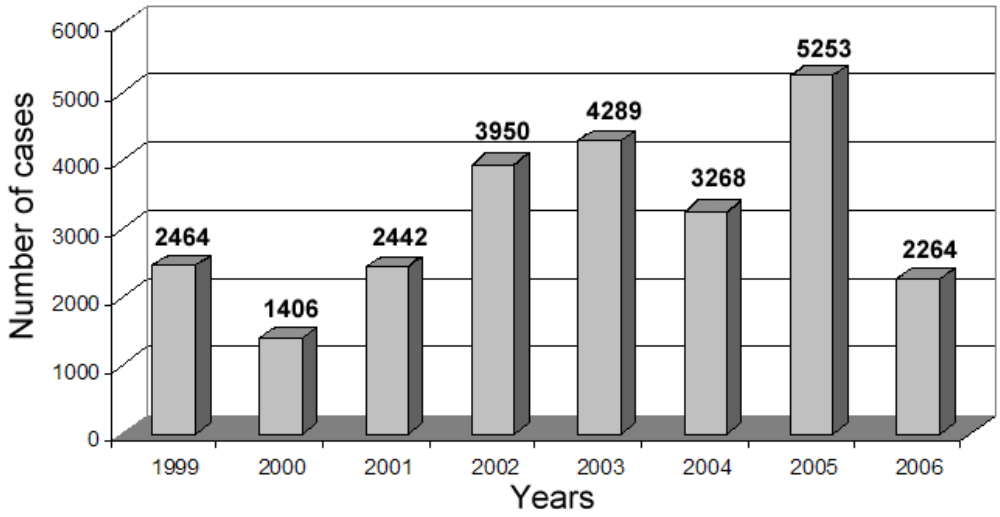

Figure 6. Dynamics of the number of animal rabies cases (total for all species) in Russia during the last 8 years.

It is well established that the main reservoirs of the rabies virus in the Russian Federation are wild carnivores. These animals are very susceptible to rabies, intensively excrete virus with saliva, are inclined to long and distant migrations, are aggressive and, in addition, have a high population density. These factors in combination with the elevated rate of generation change and relatively long incubation period assure the continuous epidemic process despite the fatality of infection. Foxes are considered to be the main wild vector of rabies in Russia, followed by raccoon dogs (Sidorov et al., 2004). Raccoon dogs have been introduced to the European part of the former Soviet Union in the 1930-1940, and became an important vector for rabies in Russian. The first cases of rabies in these animals were recorded during the winter of 1931-1932 and the first case of human rabies after being bitten by a raccoon dog occurred in 1951 (Botvinkin et al., 1981).

The existing situation with regards to rabies diagnosis within the regions of the Russian Federation does not allow any conclusions to be drawn relative to the dissemination of rabies, and therefore systematic monitoring is needed (Dudnikov, 2003a).

1.5.3. Models of rabies epidemics

Understanding the interaction between ecological dynamics, spatial spread, and evolutionary changes in infectious diseases is important, and will help in the interpretation of epidemiological patterns and provide a basis for constructing a predictive theory of disease emergence (Grenfell et al., 2004). The Kendall's threshold theorem (Kendall, 1957) states that an epidemic will occur if the contact between infective and susceptible individuals is as such that each infected individual, on average, meets and infects more than one susceptible individual. If this happens, the disease frequency begins to increase exponentially, if not – it declines exponentially. In reality, the exponential changes do not persist for a long period because the number of susceptible individuals becomes limited and there are consequent changes in the rate of contact (Bacon and Macdonald, 1980).

To predict local and spatial dynamics of the epizootics of rabies, mathematical models have been developed and are now widely used in rabies epidemiology. These models are based on population density, recorded data of rabies cases, landscape particularities, etc. (Moore, 1999; Childs et al., 2000; Smith et al., 2002; Russell et al., 2005). A simulation model was used by Thulke et al. (1999) to study the spatio-temporal

dynamics of a potential rabies outbreak in an immunized fox population after the termination of a long-term, large-scale vaccination programme with two campaigns per year: one in spring and the other in autumn. The use of integrated approaches, such as the geographical analysis of sequence variants, coupled with information on spatial dynamics is an indispensable aid to understand the patterns of disease emergence (Real et al., 2005).

1.6. Fixed strains of rabies virus

1.6.1. Laboratory strains

Two laboratory strains, the CVS (Challenge/Control Virus Strain) and PMPV (Pitman-Moore Pasteur Virus), are widely used. Both strains are considered to be derivates of the original Pasteur virus strain (Smith et al., 1993). The CVS strain has two stable variants: CVS-B2c and CVS-N2c, these differ in pathogenicity for healthy mice and in the capacity to affect neurons (Morimoto et al., 1999). These laboratory strains are used in different diagnostic tests such as the virus neutralization and focus inhibition tests, as well as for the potency testing of anti-rabies vaccines in laboratory animals.

1.6.2. Vaccine strains and anti-rabies vaccines

Since the first rabies vaccination in 1885 by Louis Pasteur (Pasteur, 1885), significant progress has been made in improving the pre- and post-exposure treatment of human rabies (Dietzschold et al., 2003). There are several types of vaccines: live attenuated, inactivated (killed), DNA-based, and vector vaccines. For the production of anti-rabies vaccines, a number of attenuated vaccine strains are employed: the Pasteur Virus (PV), Evelyn Rokitniki Abelseth (ERA), Street-Alabama-Dufferin (SAD), 3aG, Fuenzalida S-51 and S-91, Ni-Ce, SRV9, PM, Nishigahara, RC-HL, Kelev, Flury, “Shelkovo-51”, “O-73 Uz-VGNKI”, “RV-71”, “Krasnopresnenskii-85”, and the RV-97 strain (Steck et al., 1982; Fodor et al., 1994; Gruzdev and Nedosekov, 2001; Ito et al., 2001b; Borisov et al., 2002). The PV is one of the first vaccine strains; it was isolated from a rabid cow in 1882 and attenuated by multiple passages in rabbits. The SAD strain was isolated from a rabid dog in Alabama (USA) in 1935 and adapted for cultivation in the mouse brain and in the baby hamster kidney cell culture. It has two main derivates: ERA and Vnukovo-32. Several variants of the SAD strain exist: SAD-Berne, SAD B19, SAD-P5/88 etc., and also non-virulent mutants SAG-1 and SAG-2. The vaccine strains belonging to the SAD group are widely used throughout the world. One of the most

widely used oral anti-rabies vaccines is prepared from the SAD B19 strain, the high immunogenicity and relative safety of this strain has been demonstrated experimentally (Vos et al., 2000; Neubert et al., 2001).

Live attenuated vaccines are still in use in some developing countries for parenteral vaccination of animals and humans. These contain live attenuated rabies virus which has been developed in cell cultures or in live animals such as sheep. In the developed world, live attenuated vaccines are only used for the oral immunization of wild animals. Oral vaccines are widely used and several vaccine strains are used for the production of such vaccines: the SAD B19 and other SAD-strains, SAG1 and SAG2 – apathogenic deletion mutants, Vnukovo-32, and the VRG strain (Brohier et al., 1991; Vos et al., 2000). The vaccine strain RV-97 is used in Russia for producing the oral anti-rabies vaccine “Sinrab”. This strain was obtained in the FGI “Federal Centre for Animal Health” (Vladimir, Russia) from strain RB-71. The ancestor to these two strains is the strain “Sheep”, derived from the PV strain. The strain “Moscow” is also believed to be a derivate of the PV strain (Gruzdev and Nedosekov, 2001), and was used in the former USSR for producing anti-rabies vaccine. The strain RV-97 is adapted for cultivation in cell culture BHK-21 (Borisov et al., 2002).

Inactivated vaccines. Complete inactivated rabies virus particles are highly immunogenic. The vaccines based on this principle are used for the pre- and post-exposure immunization of humans and domestic animals (Dietzschold et al., 2003). The inactivated chicken embryo vaccines and vaccines based on virus cultivated in cell cultures are used for veterinary and medical purposes (Sihvonen et al., 1993, 1994, 1995; Briggs et al., 1996; Benjavongkulchai et al., 1997). Modern medical vaccines can be administered by the intradermal route (WHO, 1995; Dressen, 1997; WHO, 1997).

DNA vaccines are based on plasmid vectors expressing rabies virus glycoprotein. These vaccines have been tested for their efficiency in several animal species (mice, dogs and nonhuman primates), and it has been found that the DNA vaccine develops VNA levels and offers protection comparable with those obtained with the inactivated vaccines (Ray et al., 1997; Wang et al., 1998; Bahloul et al., 1998; Perrin et al., 1999; Lodmell, 1999; Osorio et al., 1999; Lodmell et al., 2000, 2001, 2002). On the basis of the results of the study conducted in mice, a single administration of the rabies DNA vaccine may be as effective as at least five injections of the cell-culture-derived vaccine (Bahloul et al., 2003).

Vector vaccines are based on recombinant viruses, and several viruses have been tested for these purposes. The VRG vaccine was designed on the basis of poxvirus (vaccinia virus) expressing SAD strain glycoprotein and used for oral immunization of wildlife (Wiktor et al., 1984; Brochier et al., 1990, 1991; Winkler et al., 1992; Meslin et al., 1994). The Adrab.gp - vaccine is based on the adenovirus expressing the ERA strain glycoprotein and was found capable of inducing an immune response in dogs (Tims et al., 2000). The canine herpesvirus (CHV) expressing the glycoprotein of rabies virus has also been used successfully as an anti-rabies vaccine (Xuan et al., 1998). A raccoon poxvirus (RCNV) recombinant vaccine for the immunization against feline panleukopenia and rabies has been developed and tested in cats (Hu et al., 1997). A recombinant rabies virus vaccine carrying two identical glycoprotein (G) genes (SPBNGA-GA) has also been constructed (Faber et al., 2002).

The rabies virus vaccine strain based on vectors have shown great promise as vaccines against other viral diseases such as human immunodeficiency virus type 1 (HIV-1) infection and hepatitis C, but a low residual pathogenicity remains a concern for their usage (McGettigan et al., 2003).

Plant-derived antigens can also be used for the immunization against rabies. The coat protein of alfalfa mosaic virus has been used as a carrier molecule to express the antigenic peptides from rabies virus. The *in vitro* transcripts of the recombinant virus with sequences encoding the antigenic peptides have been synthesized from DNA constructs and used to inoculate tobacco plants. The plant-produced protein (virus particles) has been purified and used for the immunization of mice, and specific anti-rabies virus-neutralizing antibodies in immunized mice have been found (Yusibov et al., 1997; Modelska et al., 1998); spinach has also been used for this purpose (Koprowski, 2002). The transgenic maize expressing the G protein of the Vnukovo strain has also been obtained and tested in mice. It was shown that the mice developed virus neutralizing antibodies which were able to provide protection of 100% against the challenge of a vampire bat strain (Loza-Rubio et al., 2007).

Oral vaccination of wildlife against rabies. Before the era of oral vaccines, the only feasible measure for controlling rabies in wildlife was the depopulation of reservoir species (Aubert, 1994); but currently rabies is the only zoonosis that can be controlled by the oral vaccination of wildlife. The idea of conducting active immunization of wildlife appeared in the last century (Baer, 1975), but many difficulties, such as the form of the vaccine, methods of distribution and uptake control, and possible residual pathogenicity

have to be surpassed. Since then, several laboratory and field trials have been conducted (Wandeler et al., 1988), and different delivery methods including vaccine traps and wool getters were designed (Winkler and Bogel, 1992; Matter et al., 1998). Initially, plastic vessels containing the vaccine were attached to chicken heads (Steck et al., 1982), but recently different types of modern vaccine baits and different meal mixtures for producing these were developed and tested (Linhart et al., 1997). The vaccine based on the strain SAD B19 is one of the most widely used in Europe: 70 million vaccine baits were used between 1983 and 1988 (Vos et al., 2000). Studies on the immunogenicity and efficacy of the SAD B19 attenuated rabies virus vaccine in foxes were conducted under laboratory conditions (Neubert et al., 2001).

Vos et al. (1999) studied the safety of the SAD B19 vaccine in 16 animal species by different administration routes; a low residual pathogenicity was observed only for certain rodent species, but transmission of the vaccine virus to control animals was not demonstrable, since no vaccine virus was detected in the saliva of the six mammal species examined. Furthermore, the genetic stability of the SAD B19 vaccine was shown through passage in neural tissue of dogs, foxes, and mice. From those results presented here on the innocuity and stability, it can be concluded that the SAD B19 rabies vaccine is suitable for the oral vaccination campaigns of carnivores against rabies (Vos et al., 1999). Nevertheless, several rabies cases have been caused by live attenuated viruses (Pastoret et al., 1999; Wandeler, 2000), so the development of new, safer vaccine strains is a very important issue.

Two mutant vaccine strains were obtained by directed mutagenesis of the strain SAD. The SAG-1 contains one nucleotide substitution, while the SAG-2 has two substitutions at amino-acid position 333 of the rabies virus glycoprotein (Follmann et al., 1996). These vaccine strains are apathogenic for adult mice inoculated by the intracerebral route (Flamand et al., 1993). The SAG-2 based vaccine was demonstrated as a safe and effective vaccine for the oral immunization of canines (Fekadu et al., 1996; Masson et al., 1996; Bingham et al., 1997, 1999; Lambot et al., 2001).

The vector-based VRG vaccine is another candidate for the oral application to immunize wild carnivores. The pathogenicity of a vaccinia recombinant virus expressing the rabies glycoprotein was tested with the red fox, wild boar, Eurasian badger, different species of mice and voles, common buzzard, kestrel, carrion crow, magpie, and jay. During the observation period, the 107 animals given the vaccine orally did not show any clinical signs (Brochier et al., 1988, 1989). Experiments have demonstrated the efficacy

of a vaccinia-rabies recombinant virus administered by the oral route in foxes. Because of its safety and heat-stability, this recombinant virus could be an excellent alternative to the attenuated strains of rabies virus currently used in the field (Brochier et al., 1990, 1991, 1996; Desmettre et al., 1990; Lambot et al., 2001). The high thermo stability of the commercially produced Raboral VRG bait allows its use during the summer for emergency vaccination campaigns (Masson et al., 1999), and is being used for the vaccination of wild raccoons in the USA (Olson et al., 2000). The VRG vaccine has also been tested as an oral vaccine in vampire bats and significant protection was observed in animals vaccinated 18-30 days before challenge (Setien et al., 1998).

Oral vaccination of wild animals has been successfully conducted in many countries: such as Austria, Croatia, Switzerland, Italy, Germany, Slovenia, Czech Republic, Slovakia, Israel, USA, Canada, Belgium, France, etc. (Steck et al., 1982; Westerling, 1989; Gram, 1996; Separovic, 1996; Schluter, 1996; Svrcek et al., 1996; Matouch, 1996; Mutinelli, 1996; Linhart et al., 1997; Olson et al., 1999, 2000; Hostnik, 2000b; MacInnes et al., 2001). The sylvatic rabies epidemic of 1988-1989 was successfully eradicated in Finland by the oral immunization of wild carnivores (Nyberg et al., 1992), and was also used in some areas of Russia. One of these vaccination areas is located at the Finnish-Russian border within the Leningrad region and the Republic of Karelia. The oral vaccination was organized in 2003 within the framework of the international Finnish-Russian collaboration program for controlling rabies in wildlife, and financially supported by the EU and the Finnish government (Metlin et al., in press).

The most common strategy for conducting oral vaccination campaigns is to use vaccine baits at a density of approximately 25 baits per square km, twice a year, during the spring and autumn, to avoid the negative influence of temperature on vaccine baits and to reach adult foxes (in spring) and juvenile foxes before they disperse (in autumn) (Aubert et al., 1994; Vos, 2003). However, further studies on the population dynamics of the red fox, the onset and progress of the reproductive season, and maternal immunity and the immune response of fox cubs to oral vaccination have shown that it was necessary to optimize the old strategy and conduct two spring vaccinations: first in March, and then before the end of May to cover young foxes (Muller et al., 1999, 2001; Vos et al., 2001).

There are two ways of distributing vaccine baits in nature: manually and by air (helicopters, airplanes). Presently, aerial distribution is widely used and special computer models have been developed to plan the distribution of vaccine baits taking into account many factors including landscape and terrain details (Thulke et al., 2004).

1.7. Molecular biology of rabies viruses

1.7.1. Antigenic characterization with monoclonal antibodies

The first mAbs of the rabies virus were obtained in 1978 (Wiktor et al., 1978). Study of rabies virus isolates with mAb directed against particular viral proteins allows antigenic characterization of the virus being evaluated and, in many cases, strain and serotype differentiation (Mebatsion et al., 1992; Delpietro et al., 1997; Nadin-Davis et al., 2000; Okoh, 2000).

With the help of mAbs directed against the different viral proteins, their antigenic and functional properties have been determined. Luo et al. (1998) studied the rabies virus glycoprotein with a panel of 35 mAbs and concluded that the G protein forms a complete antigenic structure with conformational-depended antigenic sites and epitopes. Goto et al. (2000) have used mAbs to map antigenic epitopes and to analyze the structure of the nucleoprotein antigenic sites.

Monoclonal antibodies were also used in Finland during the last sylvatic rabies outbreak. All the viruses isolated were studied using a panel of three mAbs (W-239, W-187.5, and P-41), and all induced positive reactions indicating the persistence of the Arctic antigenic variant of the rabies virus. Later, using the same panel of mAbs, 24 rabies samples collected from Estonia between 1989 and 1992 were studied; two different Arctic variants were found, one of these having the same characteristics as the Finnish isolates, the other demonstrated a unique (W-187.5 – negative) reaction (Kulonen et al., 1993).

The three mAbs mentioned above have been included in more extensive panels (up to 20 mAbs) and were used for the characterization of rabies viruses isolated in Europe (Cox et al., 1992) and different parts of the African continent (Umoh et al., 1990; Mebatsion et al., 1992). A wide range of different groups of rabies viruses was found in these studies.

Several panels of mAbs, of different origins, have been applied during the last two decades in Russia. In 1983, Selimov et al. have studied 39 rabies virus strains using a panel of 4 monoclonal antibodies; later on this work was continued and 271 field rabies

virus isolates were studied with a mAb P-41 (Selimov et al. 1983, 1994). Gribencha et al. (1989) developed a panel of 7 anti-nucleocapsid and 3 anti-glycoprotein mAbs, and these have demonstrated that individual variants of the rabies virus can be detected using this panel. Botvinkin et al. (1990) applied a panel of 39 mAbs to characterize 98 rabies viruses, which resulted in several diverse reaction patterns indicating that different antigen variants were found during those studies. In 1991, Gribencha et al. have published research studies aimed at obtaining a panel of mAbs to characterize antigenically the vaccine strain Vnukovo-32 and to compare its antigenic properties with the field rabies viruses (Gribencha et al., 1991a, b). Botvinkin et al. (2006) applied a wide panel of 74 mAbs and compared the results obtained with phylogenetic data, where it was demonstrated that the results of the antigenic studies are often in concordance to those of genetic studies.

1.7.2. Genetic characterization of rabies virus

According to the WHO Expert Consultation on Rabies (2005) it is important to conduct molecular characterization of the new field isolates of the rabies virus. Several phylogenetic and molecular-epidemiological studies on rabies have been performed during the past 10 years (Smith et al., 1992; Kissi et al., 1995; Bourhy et al., 1999, Nadin-Davis et al., 1999; Holmes et al., 2002; Kuzmin et al., 2004, Real et al., 2005). These studies have shown that the rabies viruses can be divided into two major groups, one comprising viruses isolated from terrestrial mammals and the other containing viruses isolated from bats or spillover infections from bats.

Additionally, there is a viral lineage that is closely related to the bat rabies virus but which circulates independently in raccoons and skunks, suggesting that it might represent a secondary transmission from bats. It was also found that among terrestrial mammals, rabies viruses cluster more by geographical origin than by host species, and in this case, closely related viruses infect a variety of species (Davis et al., 2006). The phylogenetic reconstruction strongly supports the hypothesis that host switching has occurred in the history of the Lyssaviruses. It has been proven that the Lyssaviruses have evolved in chiropters long before the emergence of the carnivoran rabies, probably due to spillovers from bats (Badrane and Tordo, 2001). The rabies virus is an ancient virus but it has been suggested that the current diversities in the genotype 1 of the Lyssaviruses from diverse geographical locations and different species may have started only within the last 500 years (Holmes et al., 2002).

The RT-PCR amplification, nucleotide sequencing of the different genome regions, and the subsequent genetic and phylogenetic analysis allows the determination of the genetic groups and the differentiation of the field and vaccine strains of the rabies virus. Different genome regions can be and have been used in molecular-epidemiological studies of the rabies virus. The G gene and the G-L intergenic region (pseudogene, ψ-region) are much more variable than the N gene and evolutionary pressure on individual protein coding genes within the genome varies considerably (Johnson et al., 2002a). The pseudogene region has been used for the genetic characterization of rabies viruses in several studies (Bourhy et al., 1999; Hyun et al., 2005; Nel et al., 2005). The analysis of the sequence of the nucleoprotein is considered to be adequate for reliable phylogenetic study of the rabies virus, and the additional glycoprotein gene sequence analysis is important for the characterization of antigenic and immunogenic properties of the virus (Kasempimolporn et al., 2004; Kuzmin et al., 2004).

During the last century, rabies virus was spreading to the West and South of Europe but natural barriers, such as the Vistula River in Poland, were able to limit its dissemination (Bourhy et al., 1999). Bourhy et al., (1999) have classified rabies viruses of European origin into four main groups: the NEE-group (North-East Europe), the EE-group (East Europe), the WE-group (Western Europe), and the CE-group (Central Europe). Recently, Kuzmin et al. (2004) studied a wide range of rabies viruses isolated from the territory of the former Soviet Union and classified these into five genogroups (A, B, C, D, and E). The data from that study have shown that the viruses with the same geographical origin often group together during phylogenetic analyses. The number of rabies virus variants is co-circulating in Europe, and are often associated with the red fox; also there are dog-associated rabies and the role of raccoon dogs in maintaining rabies in the Baltic countries is increasing (McElhinney et al., 2006). Mansfield et al. (2006) have demonstrated the existence of two groups within the general Arctic group: “Arctic 1” and “Arctic 2”, the latter having two subgroups and a separate “Arctic-like” group. The study of isolates from countries in the Middle East has shown the existence of few closely related groups which are different from the viruses of European and African origin; no host-dependent relations were found in this study (David et al., 2000). A molecular study of Brazilian rabies viruses has shown the presence of three main host-specific groups, especially among bat viruses and victims of vampire bats (Bernardi et al., 2005). Some authors have combined the use of mAbs and molecular methods to study the characteristics of rabies viruses (Mebatsion et al., 1993; De Mattos et al., 1996, 1999;

Nadin-Davis et al., 2001, 2003; Favoretto et al., 2002).

Most of the rabies cases in terrestrial animals and human beings are due to the genotype 1 viruses, but some cases are caused by viruses from other genotypes. Spill-over of the EBLV1 virus into the stone marten (*Martes foina*) under natural conditions has been recorded in Germany (Muller et al., 2004). Also in 1998 cases of rabies in sheep that were shown to have been infected with the EBLV-1a possibly derived from insectivorous bats were observed in Denmark (Stougaard and Ammendrup, 1998), and in 2002, a second occurrence of the EBLV-type 1 in sheep was reported (Ronsholt, 2002). Also, several human cases caused by EBLVs and ABLV strains have been reported in some European countries and in Australia (Lumio et al., 1986; McColl et al., 2000; Fooks et al., 2003; Spooner, 2003).

One of the most important issues in the field of rabies research is the identification and characterization of new genotypes. Due to PCR and nucleotide sequencing during the last ten years, one new genotype has been identified and four additional were proposed. Fraser et al. (1996) isolated a new Lyssavirus from bats in Australia, which was later identified by nucleotide sequencing as the new seventh genotype: Australian bat Lyssavirus or ABLV (Gould et al., 1998). The phylogenetic analysis of the ABLV viruses has shown that they form a monophyletic group distinct from the other Lyssaviruses, and two antigenic variants of ABLV were described (Guyatt et al., 2003). Arai et al., (2003) studied rabies virus isolated from the Lesser Mouse-eared Bat (*Myotis blythi*) in Kyrgyzstan and have suggested that it belonged to a new genotype of the rabies virus (Aravan virus). Furthermore, the Khujand virus isolated from northern Tajikistan in 2001 can be classified as a separate genotype of Lyssavirus (Kuzmin et al., 2003). Two unique viruses (Irkut and West Caucasian) have been isolated from bats in Russia and based on genetic studies it has been suggested that they belong to new genotypes (Botvinkin et al., 2003; Kuzmin et al., 2005).

1.7.3. Entire genome sequencing of vaccine strains

The entire genome sequencing of the vaccine strains provides important data relative to the rabies virus genome and allows its antigenic, genetic, and immunogenic properties to be predicted and analyzed. To conduct complete sequencing of the rabies virus genome, several techniques can be employed. Tordo et al. (1986, 1988) used cloning in plasmid vector pBR322 and sequence determination by the chain-terminating inhibitor method after inserting endonuclease restriction fragments of the cDNA inserts

into the M13 vectors. Conzelmann et al. (1990) employed the ligation of the synthetic oligonucleotide to the genomic RNA of the SAD B19 vaccine strain, after which the cDNAs obtained were ligated again with the EcoRI adaptor, and cloned into the λgt 10 phages. Ito et al. (2001b) sequenced the RT-PCR products of 13 fragments, covering almost the full-length of the viral genome to obtain the entire genome sequence of the vaccine strain RC-HL. The ligation of the synthetic SSON adaptor with T4 RNA ligase to the ends of the genomic and antigenomic RNAs was employed to obtain the 3'- and 5'-terminal noncoding regions. The PCR-amplified DNAs were then used for cloning.

The entire genome sequences of all the rabies virus vaccine strains, which are more or less used worldwide, have been determined and published in international databases. Nevertheless, the entire genome sequences of the field rabies viruses are not as common as those of vaccine strains; very few of these being currently available in public databases.

2. Aims of the study

The scope of the present study covers the genetic characterization of the field and attenuated rabies viruses and the molecular-epidemiological study of rabies in Finland and Russia. To accomplish this, the following goals had to be attained:

1. The collection of field rabies viruses from different regions of the Russian Federation, especially in the North-Western regions;

2. The development of an RT-PCR technique for rabies diagnosis and scientific purposes;

3. The antigenic and genetic characterization of the field rabies viruses from Russia, Finland (archival samples), and of the vaccine strains that are used in Russia and Finland;

4. The phylogenetic and molecular epidemiological analyses of the obtained data;

5. The entire genome sequencing, and antigenic and genetic characterization of the Russian vaccine strain RV-97 used for the oral immunization of wildlife against rabies.

3. Materials and methods

3.1. Field rabies virus isolates

Brain samples of rabid, wild, domestic/pet, and farm animals were collected from the different administrative regions of the Russian Federation (including the North-Western, Western, Southern, Caucasian, Central, and Siberian regions), Finland (archive viruses and one imported case), and Estonia (archival viruses) (*papers I, II, III and IV*).

Initially, 113 brain samples from rabid animals of different species were collected from the 11 regions of the Russian Federation (*paper I*). This study was later continued, and collectively 233 rabies brain samples from the 17 Russian regions, Finland, and Estonia were analyzed (*paper II, unpublished results*). The Russian regions close to Finland and those regions with an elevated number of recorded rabies cases were given preference during the collection of isolates. Six archival samples collected from Finland during 1988-1989 and in 2003 (imported case, *paper III*), and 5 samples collected from Estonia in 1991 (*paper I*) were included in this study.

3.2. Vaccine and laboratory strains

Two Russian vaccine strains were included in this study (RV-97 and “Sheep”): the strain RV-97 has been propagated in the baby hamster kidney cells. This strain is widely used in Russia for the production of the commercial live attenuated oral anti-rabies vaccine intended for the immunization of wild carnivores, known as “Sinrab”, FGI “Federal Centre for Animal Health”, Vladimir, and Russia (FGI “ARRIAH”). The Strain RV-97 is known to be a derivate of the strain “Sheep”, which was previously used for producing brain anti-rabies vaccines (*paper V*).

The rabies virus strain CVS-11 (ATCC VR 959) was used in the monoclonal antibody (mAb) characterization tests and in virus neutralization tests as a positive control.

3.3. Cell cultures

The murine neuroblastoma cell culture (MNA) was used for the isolation of the field rabies viruses (*papers I, II, III, and IV*), and the baby hamster kidney cell culture clone C13 BHK-21 was used for the cultivation of the vaccine strains (*paper V*).

3.4. Monoclonal antibodies

A panel of five murine anti-nucleocapsid mAbs (W-239.17, W-187.5, W-187.11.2, MW-187.6.1, and P-41) produced in the Federal Research Centre for Virus Diseases of Animals, Tuebingen, Germany, was used to study the antigenic characteristics of the field rabies viruses and vaccine strains (Cox et al., 1992) (*papers I and II*).

3.5. Laboratory techniques

3.5.1. Fluorescent antibody test

The protocols of standard techniques were used to perform the direct and indirect FAT (Dean et al., 1996; Smith and King, 1996). The fluorescent antibody and cell culture inoculation tests were performed using the rabies anti-nucleocapsid conjugate (BioRad, France), polyclonal rabbit anti-rabies FITC-immunoglobulin "ARRIAH" (FGI "ARRIAH"), and the monoclonal FITC-conjugate Centocor (Centocor Inc, USA). The rabbit anti-mouse FITC-conjugated antibodies (DAKO, Denmark) were used as secondary antibodies during the indirect FAT.

To prepare smears, glass slides and wooden sticks were used. After fixation in acetone at -20°C for at least 1h, the smears were stained with the diagnostic FITC-conjugates or mAbs in a humid chamber for 30 minutes at 37°C, washed for 10 minutes in phosphate buffered saline (PBS Dulbecco A, OXOID), pH 7.3, and then during 5 minutes in distilled water. The slides stained with mAbs were dried, stained with anti-mouse FITC-immunoglobulin, and rewashed as described above. After drying at room temperature, one drop of 50% glycerol in PBS was added to each smear and the slides were covered with cover-slips. The smears stained only with the anti-mouse FITC-conjugate were used as negative controls.

3.5.2. Cell culture inoculation test

The cell culture inoculation test was performed according to the OIE Manual (2004) with slight modifications (Kulonen et al., 1993). After preparation of 10% (w/v) brain suspension and centrifugation at 1000 g, the supernatants were filtered using disposable plastic syringe filters ("Schleicher & Schull" FP 30/0.45 CA-S). For virus isolation, 1.0 ml of the filtered supernatant was mixed with 1.0 ml MNA cell suspension at the concentration of 10^6 cells/ml in Eagle's minimum essential medium (MEM) with 10% fetal bovine serum. Diethylaminoethyl-dextran (DEAE-dextran, Sigma®, mol. wt. 500 000)

was added to all mixtures and these were then incubated at 37°C for 30 minutes, with shaking every 10 minutes and centrifuged. The cell pellet obtained was resuspended in 4.0 ml of MEM and mixed with 9.0 ml of the same medium. From the volume obtained, 6.0 ml were divided into 6 wells of a 24-well tissue culture plate (Nunclon®, Denmark) and 7.0 ml were placed in 25-cm^2 plastic culture flasks (Nunclon®, Denmark).

The plates and flasks were incubated at 37°C in 5% CO_2 atmosphere for 4 days. After incubation, the flasks were frozen, thawed to destroy the cells, and the liquid obtained was used for the next passages. At least two passages were made before staining, but in some instances more (up to 6) were done. The plates were stained with polyclonal FITC-conjugates ARRIAH and mAbs. A similar suspension made from the brain of a healthy dog was used as the negative control.

For the staining of the cell-culture plates, the culture medium was removed from the wells and 1.0 ml of 80% acetone was added to each well. The plates were incubated at 4°C for 30 minutes, the acetone was then removed and 0.2 ml of the FITC-conjugate or mAbs was added to the appropriate wells. The plates were incubated at 37°C for 30 minutes, washed with PBS for 10 minutes and then with distilled water for another 5 minutes. The FITC-conjugated rabbit anti-mouse immunoglobulin was added to the wells stained with mAbs, and incubation and washing were carried out as described above.

3.5.3. Reverse-transcriptase polymerase chain reaction

The total RNA for RT-PCR was isolated from cell-culture or brain suspensions using the “RNeasy Mini Kit” (QIAGEN®, Great Britain). The cDNA synthesis, using 5 µl of purified total RNA as a template, was carried out with the MuLV-reverse transcriptase and the 5 pmol of Random Hexamers primer (Applied Biosystems) at 37°C for 90 minutes in a volume of 20 µl.

Two primer pairs were designed for more detailed estimation of molecular-biological characteristics by PCR: the first pair (N-primers) amplifies the N-gene segment of 380 base pairs (nt 626-986), and the second pair (G-primers) 280 nucleotides (4835-5095) covering the end of the G-gene and partially the G-L intergenic region (*paper II*). The numbering of the nucleotides is according to the SAD B19 sequence (Conzelmann et al., 1990).

All PCR reactions were carried out in a volume of 50 µl. The mixtures included 5 µl of 10-fold buffer for DNA polymerase, 5 pmol of each primer, 1 U Dynazyme II of DNA polymerase (Finnzymes), and 1 µl 10 mM of dNTP’s. Thirty-five cycles of template

denaturation at 94°C for 1 minute, primer annealing at 55°C for 1 minute, and polymerization at 72°C for 1 minute were done. The products were run in 2% agarose gel and results were visualized using ethidium bromide (*papers II, III, and IV*).

3.5.4. Nucleotide sequencing

Before sequencing, the PCR products were purified by using the "MicroSpin S-400HR" columns (Amersham Pharmacia Biotech, USA). The BigDye Terminator Cycle Sequencing kit was used according to the manufacturer's instructions; the sequencing run was carried out using the 16-capillary sequencer ABI3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) at the Institute of Biotechnology, University of Helsinki, and at the Evira Virology Unit. The sequencing results were edited by using the Staden Package v.2003.0-beta (Staden et al., 2000).

3.5.5. Entire genome sequencing of the vaccine strain RV-97

The RV-97 strain used herein was obtained from the live, attenuated, oral anti-rabies vaccine intended for the immunization of wild carnivores ("Sinrab", FGI "ARRIAH"). After 1 passage in BHK-21 cell culture (clone C13), the strain was used for further genetic studies. The entire genome sequence of the vaccine strain RV-97 was obtained by joining 16 fragments (Fig. 7). The 3'- and 5'- termini of the genome were also obtained using the universal primer END_Oligo (*paper V*).

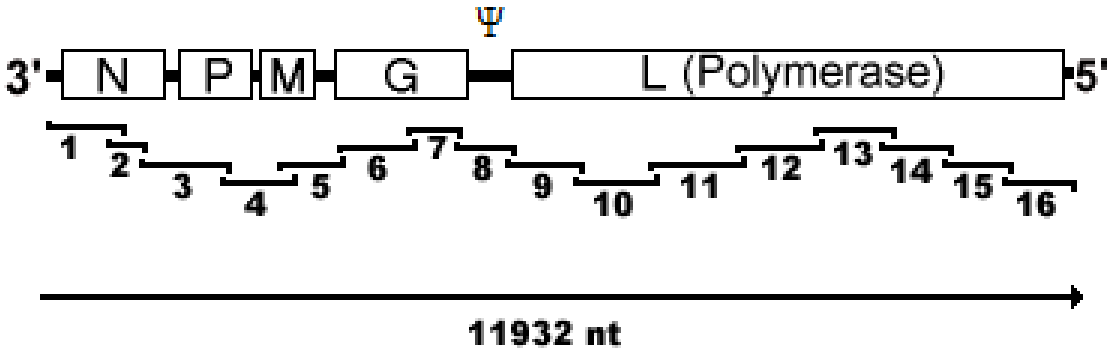

Figure 7. Schematic location of the primer pairs within the viral genome. Designations on the figure: N – nucleoprotein; P – phosphoprotein; M – matrix protein; G – glycoprotein; L – RNA-dependent RNA polymerase; Ψ – G-L intergenic region (pseudogene).

The total RNA was purified from the virus-infected cell culture suspension by using the “RNeasy Mini Kit” (QIAGEN®, cat no. 74104). The cDNA synthesis and RT-PCR were performed, as described in *paper II*, with R-primers listed in table 1 or with Random Hexamers primer (Applied Biosystems). The only difference is that polymerisation was carried out for 1.5 min. To amplify the 3’- and 5’- terminal non-coding regions, the primers 1R and 12F were used to obtain the cDNAs. Further, the RT-PCR was conducted with the primers 1R and 12F and an “END_Oligo” synthetic adaptor.

The technique for obtaining the 3’- and 5’- terminal non-coding regions is based on the reverse complementarity of the first 11 nucleotide bases at both ends of the rabies virus genome (Fauquet et al., 2005). The use of the END_oligo primer does not make it possible to evaluate the sequence of 11 nucleotide 3’- and 5’- terminals, which are considered to be conserved even between rabies and rabies-related viruses (Bourhy et al., 1989). The first 11 nucleotides at the 3’- and 5’- terminals were also conserved in all the sequences used for the phylogenetic analysis during this study, and it was presumed that the RV-97 strain contains the same sequences.

The RT-PCR products obtained were purified by using the “MicroSpin S-400HR” columns (Amersham Pharmacia Biotech, USA). The purified products were sequenced with the same primers that were used for the PCR (Table 1 in *paper V*). A BigDye Terminator Cycle Sequencing kit was used for the sequence reactions according to the manufacturer’s instructions; the sequencing was carried out using the 16-capillary sequencer ABI3100 Genetic Analyser (Applied Biosystems, Foster City, CA, USA). The sequences were assembled by using the Staden Package v. 2003.0-beta by joining 16 fragments including the non-coding 3’- and 5’- ends of genome (Staden et al., 2000).

3.6. Phylogenetic analysis

The phylogenetic analyses were performed by using the MEGA Version 3.1 program (Kumar et al., 2004). For the field rabies viruses, the minimum evolution phylogenetic trees were constructed and bootstrapped with 1000 replicates. The neighbor-joining phylogenetic trees were also built using the Kimura-2 parameter. In addition to the field strains, six rabies vaccine strains were included in the phylogenetic analysis. The sequences obtained in this study were compared with a wide variety of sequences published in the Gene Bank database, after which representatives of the genogroups created were used to construct the final phylogenetic trees. The rabies virus isolated from a bat in Colorado was used as the out-group (*papers III and IV*).

The entire genome sequence of the Russian vaccine strain RV-97 was aligned with 10 entire genome sequences of vaccine the strains obtained from the Gene Bank database. For phylogenetic analysis, the nucleotide sequences of the 5 viral genes of each strain were translated into protein sequences and joined to one sequence in the original order. The bat virus SHBRV-18 was taken as an out-group and the minimum evolution phylogenetic tree was rooted to this virus. The phylogenetic tree was bootstrapped with 1000 replicates (*paper V)*.

4. Results

4.1. FAT and cell culture inoculation test

All samples tested by the diagnostic FAT were rabies positive. The samples were further analyzed with indirect FAT on glass slides (field viruses from the Russian Federation) or in cell cultures (archival viruses from Finland and Estonia and vaccine strains). In some cases, cell culture inoculation tests and RT-PCR and nucleotide sequencing were conducted; these results were also positive.

4.2. Antigenic characteristics of field rabies viruses (papers I, II and III)

Five antigenic variants were found among the 233 virus samples tested by means of the reaction pattern with the panel of mAbs (Table 1).

Table 1. Antigenic variants of rabies virus isolates using mAbs

Antigenic variant/Region	**Genotype**	**Reaction with Mab**				
		W-239.17	**W-187.5**	**W-187.11.2**	**MW-187.6.1**	**P-41**
The results of this study						
I. Bryansk, Estonia, Finland, Kursk, Pskov, Tver	1	+	+	+	+	+
II. Estonia		+	-	+	+	+
III. Bashkiriya, Belgorod, Bryansk, Krasnodar, Kursk, Mordoviya, Novosibirsk, N.Novgorod, Orel, Penza, Tver, Vladimir, Voronezh		+	+	+	+	-
IV. Bashkiriya, Krasnodar, Mordoviya, Moscow, N. Novgorod, Ryazan, Tver, Tula, Vladimir		+	-	+	+	-
V. Tver, Moscow		+	-	+	-	-
Results from the literature						
Arctic[1]	1	+	+	+	+	+
European fox[2]	1	+	+	+	+	-
West-European[2]	1	+	-	+	+	-
Logos bat[3, 4]	2	+	-	-	+	-
Mocola[3, 4]	3	+	-	-	+	-
Duvenhage[3, 4]	4	+	-	-	+	-
EBL1[2]	5	+	-	-	-	-
EBL2[1]	6	+	-	-	+	-

[1] Schneider et al., 1985; [2] Cox et al., 1992; [3] Umoh et al., 1990; [4] Mebatsion et al., 1992.

The antigenic variant I seem to correspond to the Arctic antigenic variant, while the antigenic variants III and IV corresponded to the European fox and West-European antigenic variants, respectively (Cox et al., 1992). The antigenic variants II and V did not correspond to any of the previously found variants.

The antigenic variant I circulates mainly in the North-Western regions of Russia, Finland, and Estonia (see map*, paper I)*. The antigenic variant II was isolated only from the Estonian islands (Kulonen et al., 1993), and the antigenic variants III and IV from the Central and South-Western regions of Russia. The antigenic variant V was isolated from the Tver and Moscow regions of the Russian Federation (*papers I and II)*.

The fixed rabies virus strains can be divided into two groups according to their reaction pattern with these five mAbs: SAD B19 belongs to the antigenic group V and RV-97, «Sheep» and CVS-11 strains belong to the antigenic group III.

4.3. Development of RT-PCR tests (papers II and III)

The RT-PCR that was developed is based on the amplification of two distant regions of rabies virus genome: a fragment of the nucleoprotein gene and the G-L intergenic region. Field rabies viruses selected on the basis of the reaction patterns with mAbs and the vaccine strains SAD B19, CVS-11, RV-97, and «Sheep» were successfully amplified with the N- and G-primer pairs, producing bands of the expected sizes (Fig 8). The RT-PCR products were suitable for further automated nucleotide sequencing.

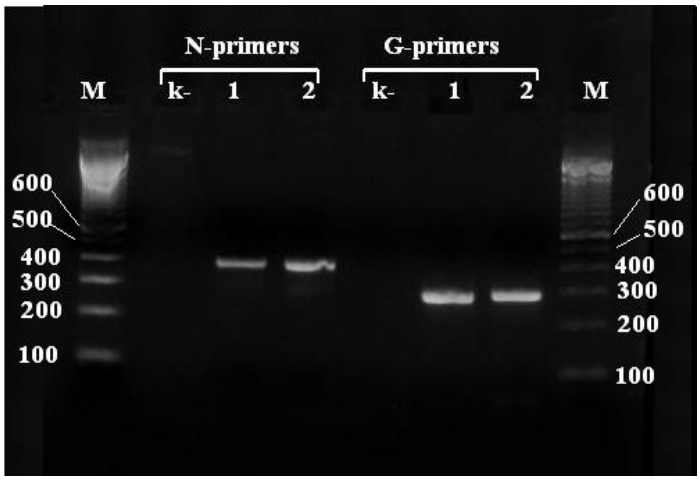

Figure 8. RT-PCR electrophoresis picture. Field rabies virus Tver-96 (line 1 N and G) and vaccine strain RV-97 (line 2 N and G). k- - negative control, M - marker.

4.4. Phylogenetic analysis and molecular epidemiology (papers III and IV)

Among the field viruses, two major phylogenetic groups, the Pan-Eurasian and Caucasian, were observed (Fig. 9). The Pan-Eurasian group was further divided into four separate subgroups. Three of them: the Eurasian, North European, and Central Russian subgroups were observed during this study. The fourth subgroup includes some European viruses classified as members of the West Europe (WE) and Central Europe (CE) groups by Bourhy et al. (1999). According to the N-gene sequence analysis, the nucleotide differences within these groups were approximately 2% and 4-7% between subgroups.

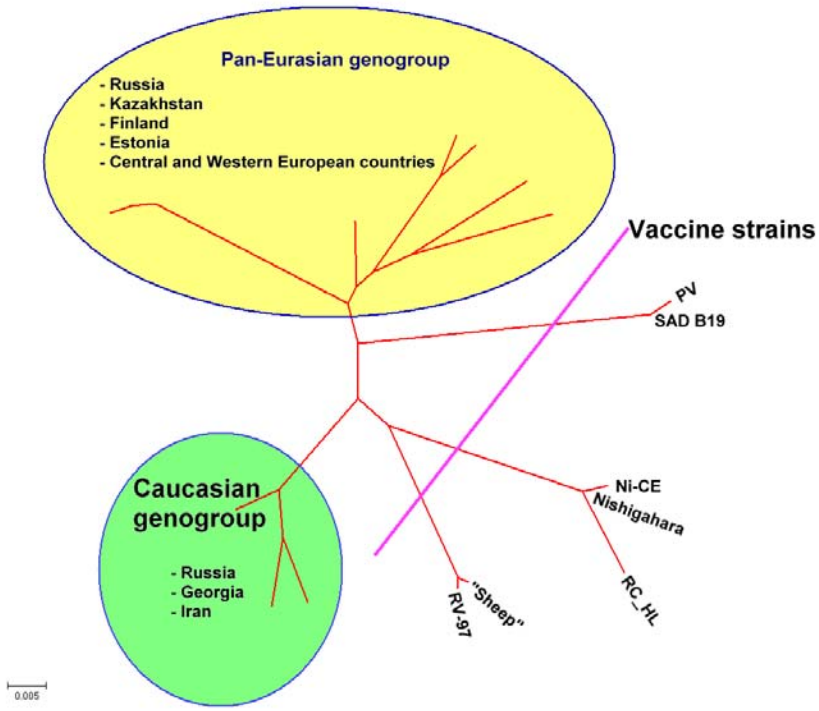

Figure 9. Minimum evolution phylogenetic tree. Phylogenetic relations of the two major genogroups and 7 vaccine strains (Metlin et al. in press). Reference sequences included: Tordo et al., 1986; Conzelmann et al., 1990, Bourhy et al., 1999; Ito et al., 2001b; Nadin-Davis et al., 2003 ; Kuzmin et al., 2004.

The “Eurasian” group comprises viruses from the European, Central, Asian, and Siberian parts of Russia, as well as from Kazakhstan (Fig. 10, *paper IV*). The “North-European” group contains viruses from Finland and Estonia, as well as from the Pskov, Bryansk, and the Leningrad regions of Russia. The viruses from the geographically close Moscow, Vladimir, Tver, and Tula regions constitute a separate “Central-Russian” group. A similar grouping was found based on the G-L intergenic region sequences. Additionally, the phylogenetical groups in most cases corresponded to antigenic variants as revealed by the mAbs.

Figure 10. Geographical distribution of the rabies viruses groups observed during this study. N-E – North-European Group (includes archival viruses from Finland, Estonia, and Leningrad region), Eurasian – Eurasian group, C-R – Central-Russian group, Cau – Caucasian group (framed). Lakes, rivers, gulfs, seas, and oceans are light blue. Arm – Armenia; Azr – Azerbaijan; Czh – Czech Republic; Est – Estonia; Geo – Georgia; Lth – Lithuania; Lva – Latvia; Mld – Moldova; Rom – Romania; Svk – Slovakia; Uzb – Uzbekistan.

The viruses isolated from the Southern part of the Krasnodar region near the Georgian border and Black Sea coast comprise the “Caucasian” group. They are closely related to the viruses from Georgia and Iran (*paper IV*). The viruses from the Caucasian

group differ from those observed in the Pan-Eurasian group by 6-7% according to the N-gene sequences, and by approximately 9% based on the G-L-intergenic region sequences.

According to both the N- and G-L-intergenic region sequences, all the vaccine strains evaluated (*paper IV*) clearly differed from the field strains and the Russian vaccine strains “Sheep” and RV-97 grouped together with the vaccine strains of Japanese origin Ni-Ce, RC_HL, and Nishigahara. The viruses from the Caucasian phylogroup seem to be the closest wild ancestor of this group (Fig. 9).

A wide range of classical rabies viruses was included in the phylogenetic analysis when the origin of the equine strain RV1904 isolated in Finland during 2003 in a horse imported from Estonia was investigated, this virus was found to be similar to the viruses previously isolated from Russia and the Baltic region. The clinical course of the disease was also described (*paper III*).

To summarize the phylogenetic grouping, we put together and compared the classifications by Bourhy et al. (1999) and Kuzmin et al. (2004), and the classification used during this study (*paper IV*) (Table 2).

Table 2. Comparison of three different phylogenetic classifications for rabies viruses isolated in the Eurasian continent

Origin of viruses	**Classifications**			
	Bourhy et al., (1999)	**Kuzmin et al., (2004)**	**This study**	
Yakutia, Tuva, Krasnoyarsk (Russia)	---	A	---	
Far East of Russia	---	B	---	
Kazakhstan, Central, Siberian and South parts of Russia	---	C	Eurasian	Eurasian phylogroup
Central and Western regions of Russia	9215HON, Hungary, ungrouped	D	Central-Russian	
North-Western and Western Russia, Finland, Estonia, Slovakia, Poland	North-Eastern Europe (NEE)	E	North-European	
Western Eastern and Central Europe	Western Europe (WE), Eastern Europe (EE) and Central Europe (CE)	---	---	
Caucuses of Russia, Georgia, Iran	---	UG	Caucasian	

*The overlapping groupings are shadowed.

It was obvious that the phylogroups proposed by different research groups overlapped in many cases, but alternatively, they supplemented each other. Some ungrouped viruses may further be classified as members of a distinct group during further research.

4.5. Entire genome sequencing and characterization of RV-97 strain (paper V)

The entire genome sequence of the RV-97 strain obtained was 11932 nt in length. Among vaccine strains, 4 different phylogroups can be found and this division is supported by the high bootstrap values (96-100). The Russian vaccine strain RV-97 forms a separate branch and seem to be more related to the Japanese group of vaccine strains (Nishigahara etc.) (Fig. 11), since it shares 92-93% nucleotide sequence identity and 95% amino-acid sequence identity with the Japanese group. The identity of the nucleotide and amino-acid sequences of the RV-97 strain with two other groups of vaccine strains (SAD/PV and American strains) is 89-90% and 94%, respectively; with the bat strain SHBRV-18 (out-group), the nucleotide and amino-acid identities were 80% and 91%, respectively.

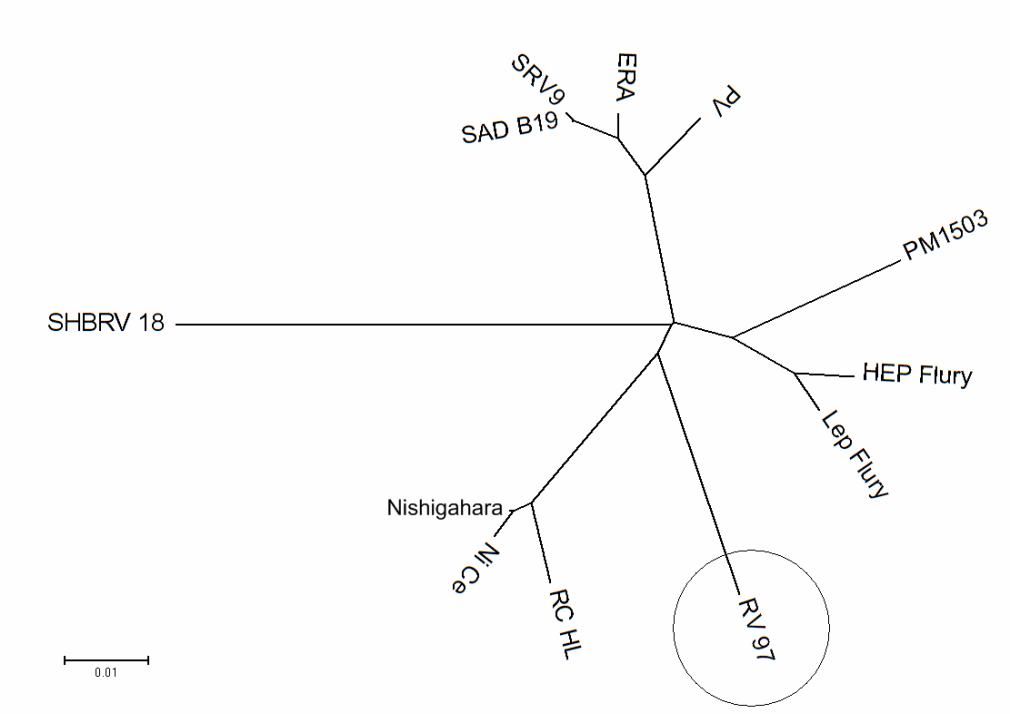

Figure 11. Minimum evolution phylogenetic tree. The phylogenetic relations of 11 vaccine strains. GeneBank accession numbers: Ni-Ce - AB128149, Nishigahara - AB044824, RC-HL - AB009663, PV - M13215, SAD B19 - M31046, SRV9 - AF499686, ERA - EF206707, Hep-Flury - AB085828, Lep-Flury - DQ099524, PM1503 - DQ099525, RV-97 - EF542830. The tree was rooted to the out-group strain SHBRV-18 - AY705373.

The vaccine strains RV-97 and SAD B19 were further compared at the amino acid level with other vaccine strains (Table 3).

Table 3. Comparison of some antigenic/immunodominant sites/epitopes of 11 vaccine strains

Strains*	**Amino acid sequence structure**
Nucleoprotein, AG site I (374-383).	
RV-97, Ni_CE, Nishigahara, RC_HL, LEP_Flury, HEP_Flury, SAD B19, SRV9, ERA, PV,	LTKTDVALAD
PM1503	LTKT**EM**ALAD
Nucleoprotein, antigenic site III (313-337)	
RV-97, SAD B19, SRV9, PV, PM1503 LEP_Flury, HEP_Flury, ERA	HFVGCYMGQVRSLNATVIAACAPHE
Ni_CE, Nishigahara, RC_HL	HFVGCYMGQ**I**RSLNATVIAACAPHE
Nucleoprotein, Immunodominant 404_418	
RV-97, Ni_CE, Nishigahara, RC_HL	AVYARIMMNGGRLKR
SAD B19, SRV9, ERA, LEP_Flury, HEP_Flury, PM1503	AVY**T**RIMMNGGRLKR
PV	AVY**T**RI**I**MNGGRLKR
Two antigenic sites of phosphoprotein in position 75-90	
RV-97, PV, ERA, SRV9, SAD B19	GKYREDFQMDEGEDPS
LEP_Flury	GKYREDFQMDEGEDP**N**
HEP_Flury	GKYREDFQM**N**EGEDP**N**
PM1503	GKYREDFQM**G**EGEDP**N**
Ni_CE	G**RHQ**ED**P**QMDEGEDPS
Nishigahara, RC_HL	G**RHQ**EDFQMDEGEDPS
Immunodominant cytotoxic T epitope of phosphoprotein in position 191-206	
RV-97	EEDDLSAEAEITHQIA
LEP_Flury, SAD B19, SRV9, PV, PM1503, Ni_CE, Nishigahara, RC_HL	EEDDLS**V**EAEI**A**HQIA
HEP_Flury	**G**EDDLS**V**EAEI**A**HQIA
ERA	E**K**DDLS**V**EAEI**A**HQIA
Structure of antigenic epitope I of Glycoprotein in position 231	
RV-97, LEP_Flury, HEP_Flury, PM1503, Ni_CE, Nishigahara, RC_HL, ERA, SADB19, SRV9, PV	L
Antigenic site II of Glycoprotein in position 34-42 and 198-200	
RV-97, HEP_Flury, ERA, Ni_CE, Nishigahara, PV, SAD B19	GCTNLSGFS KRA
LEP_Flury, PM1503	GCTNLS**E**FS KRA
RC_HL	GCTNLSGFS KR**V**
SRV9	**E**CTNLSGFS KRA
Antigenic site III of Glycoprotein in position 330-338	
RV-97, LEP_Flury, PM1503, Ni_CE, Nishigahara, RC_HL, ERA, SAD B19, PV	KSVRTWNEI
HEP_Flury	KSV**Q**TWNEI
SRV9	KSV**S**TWNE**V**
Antigenic epitope VI of Glycoprotein in position 264	
RV-97, LEP_Flury	H
HEP_Flury, PM1503, Ni_CE, Nishigahara, RC_HL, ERA, SAD B19, SRV9, PV	**R**
Antigenic site “a” of Glycoprotein at position 342-343	
RV-97, LEP_Flury, HEP_Flury, PM1503, Ni_CE, Nishigahara, RC_HL, ERA, SAD B19, SRV9, PV	KG

The analysis of the structure of the immunodominant 404-418 epitope of the nucleoprotein shows that the RV-97 strain is different by 1 amino acid from the strain SAD B19. The structure of immunodominant cytotoxic T epitope of the phosphoprotein at position 191-206 of RV-97 seems to be unique and different from the structures of the other vaccine strains by at least two amino acids.

Because the glycoprotein is the major antigen of the rabies virus, we analyzed the major immunodominant site mapped at position 222-332 of the mature glycoprotein (Johnson et al., 2002b). The structure of the antigenic epitope I within the framework of the major immunodominant site is conserved among the vaccine strains (*paper V)*. The antigenic epitope VI is different from the RV-97 strain and most of the other vaccine strains. Furthermore, part of the antigenic site III seems to be conserved among the vaccine strains, only two of them; SRV9 and HEP_Flury, having different amino acids at position 333, serine and glutamine, respectively. All the other strains have arginine at this pathogenicity-dependent position. There are three sites of complete differences between the strain RV-97 and other strains: serine at position 222 in RV-97 and alanine at all other strains, serine in RV-97 at position 253 and proline in all the others, and isoleucine at position 318 in RV-97 and phenylalanine in all the others.

5. Discussion

Rabies is a serious veterinary and public health problem in Russia and in some bordering countries. Finland was declared rabies-free in 1991. Since the last outbreak of rabies in 1988-1989, the spread of this disease has been prevented by vaccinating cats and dogs, and by the oral immunization of wild carnivores covering the region of Finland's south-eastern border with Russia (Nyberg et al., 1992). Since then, three imported rabies cases were recorded in this country (horse, in 2003, human and dog in 2007 (*paper III;* Kallio-Kokko H., personal communication; communication of the Ministry of Agriculture and Forestry of Finland). Because rabies is an important problem for both Finland and Russia, the Finnish-Russian collaboration on rabies control was started and all the studies described herein were conducted within frames of this collaboration.

The monoclonal antibodies allow the detection of the antigenic variants and, in many cases, the typing of rabies virus strains. Over the last 20 years, several panels of mAbs have been used in Russia (Selimov et al., 1983; Gribencha et al., 1989; Botvinkin et al., 1990). This study covers several regions of Russia, as well as Finland and Estonia. More than 200 rabies virus isolates were collected for typing by using a panel of 5 antinucleocapsid mAbs. The Russian regions, adjacent to Finland and regions having a high number of recorded rabies cases were given preference for the collection of isolates. The monoclonal antibodies used in our experiment have been used over the last decade for the antigenic characterization of Lyssaviruses of European and African origin (Schneider et al., 1985; Umoh et al., 1990; Cox et al., 1992; Mebatsion et al., 1992).

Five different antigenic variants of rabies virus isolates have been identified. The isolates reacted in the same manner as the Arctic antigenic variants isolated within the Russian Federation, Finland, Estonia, and Norway (Schneider et al., 1985; Kulonen and Boldina, 1993). The variant "II", originally isolated by Kulonen and Boldina (1993), has been tested with a more extensive panel of antinucleocapsid mAbs. A previously unknown rabies virus type was isolated from foxes in the Tver Region (Staritsky district) and in the Moscow region of the Russian Federation (*papers I and II and unpublished results)*. Some fixed rabies viruses such as the SAD, ERA etc. show the same reaction pattern (Cox et al., 1992).

The antigenic variant IV was found to be prevalent in the central regions of Russia and the antigenic variant III was predominant within the South-Western regions. The antigenic variant I was found mostly in the North-Western regions of Russia as well as in

Estonia and Finland, but also in the Kursk and Bryansk regions located in the Western part of Russia. The unique antigenic variant V was only found in the Tver and Moscow regions, located in the central part of Russia, where several rabies virus types were isolated.

A positive reaction with the P-41 mAb was considered to be the main characteristic of the Arctic antigenic variant. During the last sylvatic rabies epidemic in Finland, only the Arctic antigenic variant was found. The same virus type exists in continental Estonia as well as in the Pskov, Tver, Kursk, and Bryansk regions of the Russian Federation. The Pskov and Tver regions are adjacent to Estonia, but a very pronounced mixture of different rabies virus types was found only in the Tver Region. Selimov et al. (1994) have identified P-41 positive isolates collected from Estonia, Belarus (the Minsk region), the Leningrad, Pskov, Kaliningrad regions, and the Kola Peninsula (the Murmansk region) during 1969-1991.

It was found, that the Russian vaccine strain RV-97 demonstrated similar reaction pattern to the mAbs to the antigenic variant III, while the SAD B19 strain reacted similarly to the antigenic variant V.

Unfortunately, the panel of mAbs used in this study could not be used to differentiate between the field viruses belonging to the antigenic variants III and V from the vaccine strains RV-97 and SAD B19; other techniques would have had to be employed for this purpose.

At present, the PCR and nucleotide sequencing are more widely used for the molecular studying of rabies viruses than monoclonal antibodies. The PCR developed in this study is in use at Evira for diagnostic purpose, and also a laboratorial ante mortem diagnosis was made with the aid of this technique (human case imported from Philippines to Finland in 2007). In this study, the groupings obtained with the monoclonal antibodies in many cases correlated with the phylogenetic grouping. The nucleotide sequences obtained during this study have been deposited in the public database Gene Bank.

Finland was declared rabies-free since 1991, but in 2003 a case of rabies in an imported horse was recorded. There has been a considerable amount of exportation and importation of horses between Estonia and Finland, and between Russia and Finland, but cases of imported rabies have not been previously diagnosed in Finland. The incubation period of the rabies virus can vary considerably from a few days to several months or even years (Baer 1991). Rabies in horses must always be differentiated from other infectious diseases, such as pseudo rabies (Aujeszky’s disease), listeriosis etc., as well

as from non-infectious diseases (e.g., colic and intoxication). The occurrence of this disease in an animal imported from a rabies-affected country is highly suggestive of an imported case of rabies. The monoclonal antibody characterization of the equine virus RV1904 showed a reaction pattern typical of the Arctic viruses. The Arctic viruses are very common in Estonia and some Western regions of Russia, where rabies is endemic in wild and domestic animals. The virus isolated from this horse was genetically characterized and was found to be a either member of the North-East European group (NEE) (Bourhy et al., 1999) or the E group (Kuzmin et al., 2004), which are clearly overlapping. Presently, the Finnish Ministry of Agriculture and Forestry recommends that horses traveling to and from a rabies-affected area should be vaccinated. Imported dogs and cats have to be certified to show that they have developed ≥0.5 IU/ml anti-rabies virus-neutralizing antibodies (Regulation No 998/2003/EC**,** OIE, 2004). This antibody level is considered to be adequate to protect the animal against rabies infection. Thus, quarantine of animals with an unknown vaccination status would be a valuable measure in preventing the introduction of rabies.

The molecular epidemiological studies help to localize the origin of a virus and make it possible to develop rabies control programs, and prevent the introduction of the rabies infection into rabies-free areas. Two main phylogenetic groups were established among the field viruses studied: Pan-Eurasian and Caucasian. The general Pan-Eurasian group comprised four subgroups, designated as the Eurasian, North-European, and Central-Russian subgroups, which corresponded to the phylogenetic groups C, D, and E published by Kuzmin et al. (2004), but the WE/CE group of Bourhy et al. (1999) did not overlap with any of these groups. In our studies we prefer the usage of geographical nomenclature as it is more informative and gives key features of basic geographical locations of the phylogenetic group (Table 2).

The Eurasian subgroup comprises viruses from Siberia (Novosibirsk) to the Western (Belgorod) and South-Western (Krasnodar) regions of the Russian Federation. The viruses from Omsk, Altai (Russia), and Kazakhstan, previously classified by Kuzmin et al. (2004) as members of group C, also belong to this group. This group seems to be the most widespread in Russia (Fig. 10, *paper IV)*.

The viruses from the North-European group were positive for the P-41 mAb, indicating the Arctic antigenic group origin. The North-European group includes viruses from Finland and Estonia, in addition to the Pskov, Bryansk, and Leningrad regions. It is

clear that viruses circulating in Estonia and the Pskov region of Russia are very similar.

Previous antigenic and genetic studies have confirmed that the isolates responsible for the Finnish outbreak (1988-1989) were related, but not identical to the viruses isolated from the Baltic region (Kulonen et al., 1993; Bourhy et al., 1999; Johnson and Fooks, 2005). It may be suggested that rabies has gradually moved from the Russian/Estonian territories after prolonged circulation among local hosts before being detected. The causative agents included in this study belong to the North-European group, which corresponds to the group E of Kuzmin et al. (2004) and to the group NEE of Bourhy et al. (1999).

Several explanations, such as animal-to-animal transfer, human-mediated transfer (e.g., by railways), or the movement of infected animals across the frozen sea have been suggested (Johnson and Fooks, 2005). We report a close phylogenetic relationship among viruses isolated from Finland during the period 1988-1989 and in 2003, Estonia between 1989 and 1990, the Leningrad region (Russia) in 1990, and from the Pskov region (Russia) in 2002. Two ways of transmission seem to be more probable: gradual animal-to-animal terrestrial movement via/from the Leningrad region or across the frozen surface of the Gulf of Finland directly from the Estonian territory. The last explanation seems to be more probable because most cases were found on the Western side of the Kymi River and one case was found on an island on the southern rim of the coastal ice (Nyberg et al., 1992). Rabies could still be reintroduced into Finland by imported animals, as occurred in 2003 and in 2007, but due to the annual oral vaccination campaigns on both sides of Russia's south-eastern border with Finland, the development of major sylvatic epidemic in south-eastern Finland seems unlikely.

The Central-Russian group comprises viruses mostly from the Central part of Russia and seems to overlap with group D of Kuzmin et al. (2004). The viruses from these regions clearly differ even from those isolated from the bordering regions, such as the N. Novgorod region. The Oka and Volga rivers are considered as limiting factors for virus circulation, by separating Moscow and Vladimir and parts of the Ryazan and Tver regions from the other regions. Rivers significantly limit the extensive movement of wild carnivores and allow the independent evolution of viruses in distinct regions. During the last century, some barriers, such as the Vistula River in Poland, were able to restrict the spread of rabies from Eastern to Western and Southern Europe (Bourhy et al., 1999). However, this barrier is not absolute and animals can easily cross rivers in several ways (across the ice in winter when the water is frozen, over bridges, or by swimming).

Nevertheless, the major rivers remain an important limiting factor for the development of rabies epidemics and this must be taken into account when anti-rabies programs, especially oral-vaccination campaigns are being developed.

The mountains are also capable of limiting the circulation of the rabies virus. In our study, viruses from the north-western part of the Krasnodar region were clearly different from those viruses isolated in the area located on the other side of the Caucasian Mountains, near the border of Georgia. This area is separated from the rest of the territory in the Krasnodar region by the Caucasian Mountain Range. Some viruses from Iran (Nadin-Davis et al., 2003) and from Georgia (Kuzmin et al., 2004) belong to this phylogroup.

All the field viruses studied herein clearly differed from the vaccine strains, and also from the 96_2002 virus that was isolated in the Tver region and had a mAb reaction pattern similar to some vaccine strains of the SAD group. The oral vaccination of wild carnivores with different types of live-attenuated vaccines has been conducted in that region so that the recombination between field and vaccine strains can be theoretically possible. Although the recombination rate of the Lyssavirus is low, there are data suggesting that this may occur (Chare et al., 2003). The Russian, Estonian, and Finnish field rabies viruses and the Russian vaccine virus strains were analyzed phylogenetically on the basis of two distant genome regions, and basically the grouping of viruses was similar in both regions; these results provide no evidence to support the idea that the recombination between field viruses and vaccine strains occurred. However, these findings indicate that the vaccines used in the region were stable and did not revert back to their native pathogenic form.

A specific host for the phylogenetic groups could not be clearly defined during this study because the virus members of the phylogroups that were determined were found in both wild and domestic/farm animals. However, the viruses from the North-European group are mostly maintained in red foxes and raccoon dogs (Nyberg et al., 1992; Bourhy et al., 1999; Johnson and Fooks, 2005), and are transmitted to domestic and farm animals. The Caucasian phylogenetic group may also be associated with dogs, but further studies have to be conducted to support this statement.

The entire genome sequence analysis allows characterization of the genetic properties and phylogenetic relations at a very reliable level. It is especially important for the strains used for the production of oral anti-rabies live attenuated vaccines, because several rabies cases caused by such vaccines in wildlife have been reported (Pastoret

and Brochier, 1999). The vaccine strain SAD B19 is widely used for the production of live attenuated vaccines in Europe for the immunization of wild carnivores against rabies (Artois, 1992; Vos et al., 2000; Neubert et al., 2001). In Russia, the vaccine strain RV-97 is used for the production of anti-rabies vaccine for wild animals (Borisov et al., 2002).

Several techniques have been previously used by different research groups for the entire sequencing of rabies virus genome: cloning into plasmid vector (PV strain, Tordo et al., 1986), cloning in λgt 10 phages (SAD B19 strain, Conzelmann et al., 1990), sequencing RT-PCR products, and ligation of synthetic adaptor to obtain 3'- and 5'-terminal non-coding regions (RC-HL strain, Ito et al., 2001b). In this study, we used a technique similar to the methods described by Ito et al. (2001b) and by Bouhy et al. (1989). Sixteen overlapping fragments of the genome were obtained with RT-PCR, and subjected to automated sequencing. The technique for obtaining the 3'- and 5'- terminal non-coding regions is proposes using the universal synthetic adaptor "END_Oligo" for both ends in combination with the first reverse and last forward primers using the RT-PCR technique to obtain the 3'- and 5'-termini. The inverse complementarity of the first 10 nucleotide bases at both ends (Virus Taxonomy, 2005) of the rabies virus genome allows this simple and fast technique to be used. Unfortunately, the use of the END_oligo primer does not make it possible to evaluate the sequence of 11 nucleotides at the 3'- and 5'- terminals, which are considered to be conserved even between rabies and rabies-related viruses (Bourhy et al., 1989). The first 11 nucleotides at the 3'- and 5'- terminals are also conserved in all the sequences used for phylogenetic analysis during this study, and it was presumed that the RV-97 strain contains the same sequences.

The vaccine strain RV-97 was found not to be a member of well-known phylogenetic groups, such as the SAD/PV (SAD B19, PV, etc.), American (Hep/Lep-Flury, PM) or the Japanese (Nishigahara, RC-HL and Ni_Ce) groups, but it was more closely related to the last one of these. It was assumed that the Russian rabies virus vaccine strain RV-97 originates from the PV-strain (PV→ "Sheep" → RB-71 → RV-97; Gruzdev and Nedosekov, 2001), but this seems unlikely on the basis of data obtained from this study. Also another derivate of the PV strain, – "Moscow", was used in the former USSR for the production of anti-rabies vaccines. The analysis of additional Russian strains could be of value when searching for the nearest common ancestor of the Japanese vaccine strains and the RV-97 strain.

Most of the amino acid differences found in this study are located outside the mapped antigenic and immunodominant regions. However, six of the eight known

antigenic sites (epitopes) of the G protein are conformational, and any amino acid changes within close proximity to these epitopes could potentially affect the folding of the protein (Tordo, 1996).

Two amino acid differences were found at positions 197 (alanine in RV-97, valine in the SAD B19) and 202 (threonine in RV-97, alanine in SAD B19) inside the immunodominant cytotoxic T epitope of the P protein. The structure of the two antigenic sites of the P protein is identical for the RV-97 and SAD B19 strains, but the RV-97 strain has a unique structure of the immunodominant cytotoxic T epitope of the P protein at position 191-206. Two amino acid differences were found at positions 197 (alanine in RV-97, valine in SAD B19) and 202 (threonine in RV-97, alanine in SAD B19). Alanine and valine are neutral, nonpolar, and hydrophobic and we assume that this difference does not have a major influence on the immunogenic properties of the strains. However, valine has a larger volume (105) and surface area (160), which may be important. The change from alanine to threonine at position 202 may be more important, because of the differences in the properties of these two amino acids, as was discussed for the N protein.

The structure of the antigenic site of the M protein at position 1-72 is different in the RV-97 and SAD B19 strains, and 11 amino acid changes were found during this study. The amino acid changes at positions 7, 22, and 44 of the M protein can be considered homologous, because the amino acids changed at the positions mentioned have similar chemical properties. Other changes may be relevant for differences in the antigenic properties of M protein.

Nevertheless, only the N and G proteins are generally considered to be the most important for immunogenicity. The RV-97 strain has alanine and the SAD B19 strain possesses threonine located at position 407 of the nucleoprotein. Both of these amino acids are neutral but have major differences in volume, surface area, and polarity (alanine; volume 67, surface area 113, nonpolar; threonine: volume 93, surface area 146, polar).

The antigenic properties of the glycoprotein were mostly compared between the RV-97 and SAD B19 strains, because both of them are used for the production of live attenuated oral vaccines. Fifty-four aa changes (almost 10% divergence) took place in the glycoprotein of the RV-97 strain when compared to the SAD B19 strain. Eight of these alterations are located within the major immunodominant epitope mapped at position 222-332 of the mature glycoprotein (Tordo, 1996). The first two changes at

positions 222 and 242, from alanine to serine and back, may be important, particularly for the specificity of antibodies than for the immunogenicity of the glycoprotein: both of these amino acids are neutral, but serine is a polar amino acid and alanine is non-polar, and consequently alanine is 5 times more soluble. However, these changes are reciprocal within twenty amino acids and the immunogenicity of this part of the sequence can be similar. The mutation of serine to proline at position 253 is considered important because they have similar properties, but proline may alter the rigidity of the aa chain. The mutation of proline to serine at position 253, and of lysine to glutamine at position 256 may, be very important because proline is more hydrophobic than serine and lysine compared with glutamine, and is a very soluble amino acid. Moreover, the basic amino acid residues, such as lysine, are known to be essential for immunogenicity (Yi et al., 2005). The mutations of arginine to histidine at position 264, arginine to lysine at position 278, and of phenylalanine to isoleucine at position 318, seem to be conservative because these amino acids have similar properties. All of the vaccine strains analyzed during this study contain leucine at position 231, which represents the antigenic epitope 1 of the glycoprotein. The RV-97 and SAD B19 strains also contain identical structures at the antigenic site 2 (position 34-42 and 198-200), antigenic site 3 (position 330-338) and “a” (position 342-343). But the antigenic epitope VI at position 264 changes from histidine in RV-97 to arginine in SAD B19, both of these amino acids are basic but arginine has a greater volume and surface area.

Because amino acid changes can potentially affect the folding of proteins which can have an influence on the conformational antigenic sites (epitopes) (Tordo, 1996; Moore et al., 2005), the differences determined can theoretically influence the immunological characteristics of the RV-97 strain. Further studies need to be conducted to support that because immunogenicity and pathogenicity of rabies virus have complex nature and is not fully understood yet.

To differentiate between the field rabies viruses and vaccine strains, several techniques can be employed: sequencing of RT-PCR products, the cleavage of RT-PCR products with restriction enzymes, and real-time PCR. It was shown that the RT-PCR products of the G-L intergenic region of most of the vaccine strains are longer than those of the field viruses. The restriction enzyme technique and real-time PCR are now under development within the framework of the present project and the results of this study will soon be published. The preliminary studies have shown that these techniques to be fast, simple, and informative.

The data included in this thesis can be used when planning the utilization of vaccines in different geographical regions. The wide genetic heterogeneity of field rabies virus strains was also verified in this study and indicates that we should remain alert to the potential spread of rabies and that international rabies-control programs must be established in rabies-affected areas. Data from this study has shown that it is necessary to continue investigating the genetic characteristics of the field and vaccine strains of rabies virus in order to determine their similarities and differences.

6. Concluding remarks

Studies on rabies are considered to be very important by the international scientific community. Recently, several new genotypes were posited among Lyssaviruses, and further studies in this field are acknowledged. Genetic studies also help in the development of effective rabies-control programs, especially in wildlife.

A diagnostic RT-PCR was developed during this study and is now in use as a supplementary method at Evira to support rabies diagnosis by FAT and cell-culture inoculation tests. With the help of a panel of mAbs, we found a great antigenic variability of the field rabies viruses in the Russian Federation. Further, genetic studies have helped to identify two major genetic groups. The Pan-Eurasian group comprises at least 4 subgroups with viruses from different parts of Europe, Russia, and Kazakhstan. The Caucasian genetic group has not been previously defined. It comprises viruses from the Krasnodar region, an isolated region near the Georgian border, and also previously ungrouped viruses from Georgia and viruses from Iran. The distribution of the viruses from this group must be studied in the future, because other countries could be included in the area of circulation of the Caucasian strains. In the future, the Caucasian may be renamed the Trans-Caucasian group if viruses from other Caucasian countries are included.

Currently, all the most widely used rabies vaccine strains have been sequenced and these sequences are available at the Gene Bank. In the present study we obtained the complete genome sequence of the vaccine strain RV-97 used in the Russian Federation for the production of the oral anti-rabies vaccine used to immunize wild carnivores. We also analyzed its antigenic and genetic properties in comparison with other rabies virus vaccine strains and compared the *in vivo* immunogenicity of the RV-97 and SAD B19 in caged silver foxes. The differentiation between the field and vaccine strains of the rabies virus seem to be very important and several methods can be used to differentiate between these strains. In our study we used nucleotide sequencing for this purpose as it is very reliable, but laborious and very expensive. A more modern method, such as the restriction enzymes testing and real-time PCR would be a valuable asset for this purpose in the future.

The data presented in this study can be used when planning rabies control programs in different geographical regions. The viral sequences obtained during this study are available to the public and can be used by other research groups in molecular-epidemiological studies.

7. Заключение (concluding remarks in Russian)

Научно-исследовательская работа в области диагностики, профилактики и борьбы с бешенством признана особо значимой международным сообществом. За последние годы было открыто несколько новых генотипов лиссавирусов и дальнейшие исследования в этой области имеют большое значение. Кроме того, результаты молекулярно-биологических исследований используются при разработке программ по борьбе с бешенством, особенно среди диких животных.

В рамках данной работы был разработан метод диагностики бешенства основанный на обратно-транскриптазной полимеразной цепной реакции (ОТ-ПЦР). Данный метод используется в настоящее время в Финском Агентстве по Безопасности Продуктов Питания (Evira) в качестве дополнительного диагностического метода наряду с реакцией иммунофлюоресценции и выделением вируса бешенства в культуре клеток. Кроме того, он также использовался в нашей работе для получения ПЦР-продуктов с целью их последующего нуклеотидного секвенирования.

С помощью панели моноклональных антител была изучена антигенная вариабельность изолятов вируса бешенства выделенных на территории Российской Федерации. Последующие генетические исследования позволили выявить две основные генетические группы. Пан-Евроазиатская группа включает как минимум 4 подгруппы, в которые входят вирусы из Казахстана и различных Европейских стран, включая Россию. Доступность референтных нуклеотидных последовательностей в международных базах данных позволила подтвердить достоверность наших исследований. Кавказская генетическая группа ранее не была обозначена как таковая. В неё входят вирусы выделенные в Краснодарском крае вблизи границы с Грузией, а также раннее выделенные вирусы в Грузии и Иране. В будущем эта группа может быть переименована в Транс-Кавказскую в случае если вирусы, принадлежащие к данной группе будут выявлены и в других странах кавказского региона.

С появлением в международных базах данных большого числа полных последовательностей геномов вакцинных штаммов вируса бешенства, общепризнанной стала обязательность секвенирования полного генома всех используемых вакцинных штаммов. В результате проведенных нами исследований был получен полный геном вакцинного вируса бешенства, штамм РВ-97,

используемого в Российской Федерации для производства оральной антирабической вакцины. Был проведен сравнительный анализ генетических и антигенных свойства этого штамма в сравнении с другими вакцинными штаммами вируса бешенства.

Дифференциация полевых изолятов и вакцинных штаммов вируса бешенства имеет большое значение. С этой целью используются несколько лабораторных методов. Кроме общепризнанного референтного метода – нуклеотидного секвенирования фрагментов генома, могут применяться и другие методы, такие как, обработка ПЦР-продуктов рестриктазами и ПЦР в реальном времени.

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