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INNOVATIVE TECHNOLOGIES FOR THE DETECTION AND CONTROL OF RABIES

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RIASSUNTO

La rabbia è un encefalomielite acuta causata da virus a singolo filamento di RNA a polarità negativa appartenenti al genere *Lyssavirus*, parte della famiglia *Rhabdoviridae*. Il virus si trasmette all'uomo da animale infetto, generalmente mediante morso. Il virus infetta il sistema nervoso centrale, mediante colonizzazione centripeta delle cellule nervose. L'infezione virale del sistema nervoso centrale è fatale nella maggioranza dei casi e dall'inizio della sintomatologia, che può essere catalogata in due forme: rabbia paralitica che determina la paralisi progressiva della vittima, o rabbia furiosa caratterizzata da aggressività, ipersalivazione ed insufficienza respiratoria acuta.

Benchè le organizzazioni internazionali abbiano classificato tale malattia come una zoonosi altamente prioritaria, la rabbia è ancora trascurata nella maggiorparte dei Paesi in via di sviluppo. L'Organizzazione Mondiale della Sanità (OMS) calcola che ogni anno almeno 10 milioni di persone ricevano il trattamento post-esposizione e stima una perdita di almeno 55.000 vite, la maggiorparte bambini in Asia e Africa (Knobel *et al.*, 2005). Queste stime necessiterebbero sicuramente ulteriori conferme, alla luce dei risultati di diversi studi di *modelling* dei casi di rabbia altamente indicativi di un'incidenza superiore della malattia (Ly *et al.*, 2009, Mallewa *et al.*, 2007).

Nonostante le gravi implicazioni per la salute pubblica, la rabbia rimane nella maggiorparte dei casi, sottostimata e poco diagnosticata nelle aree endemiche. A tal proposito, lo sviluppo di metodiche diagnostiche innovative, cosiccome lo studio delle dinamiche evolutive dei virus circolanti nelle specie *reservoir* della malattia, sono due strumenti utili sia per aumentare la sorveglianza sulla malattia sia per pianificare suoi interventi di controllo.

Il candidato ha sviluppato due test biomolecolari per la diagnosi della rabbia (One-Step RT-PCR) e la sua tipizzazione mediante l'utilizzo del pyrosequenziamento. La tecnica del pyrosequenziamento è una metodica alternativa al sequenziamento Sanger, che si basa sull'identificazione di una molecola di pirofosfato nel processo di sintesi del DNA (Nyren., 2007). Le due metodiche sono state sviluppate su due geni differenti del virus rabbia, N e G (codificanti per la nucleoproteina e per la glicoproteina rispettivamente). Nel primo caso, il protocollo ha permesso di identificare la specie virale responsabile dell'infezione, nel secondo

invece di riconoscere la presenza di un virus vaccinale da un virus di campo. In particolare, la seconda metodica è stata applicata ai campioni risultati positivi per virus rabbia durante la recente epidemia in Nord Est Italia (2008-2011), durante la quale si è proceduto a vaccinazione orale delle volpi, mediante la distribuzione di esche vaccinali contenenti vaccino vivo attenuato.

Le dinamiche epidemiologico-molecolari e le dinamiche evoluzionistiche dei virus rabbia circolanti nella regione centro-occidentale della Africa e più recentemente in Italia sono state affrontate durante la scuola di dottorato dal candidato. Le analisi filogenetiche si sono avvalse dei metodi massima verosimiglianza (programmi PAUP* e PhyML) (Wilgenbusch & Swofford, 2003, Guindon & Gascuel, 2003). Sono state inoltre analizzate le sequenze ottenute mediante Bayesian Markov Chain Monte Carlo (MCMC) disponibile nel programma BEAST (Drummond & Rambaut, 2007) per calcolare le dinamiche evolutive dei virus.

Nel primo caso sono stati analizzati i virus circolanti nella regione centro-occidentale Africana. Lo studio ha dimostrato che i virus della rabbia circolanti nella popolazione canina dell'Africa Sub-Sahariana Occidentale e Centrale appartengono ad uno specifico sublineaggio detto Africa 2. Le analisi hanno dimostrato come questo specifico lineaggio sia stato verosimilmente introdotto in Africa in epoca relativamente recente (molto probabilmente 1845) e verosimilmente mediante il movimento di animali associati all'uomo (p.e. durante la colonizzazione francese della regione). Una forte caratterizzazione in cluster genetici circoscritti è inoltre indicativa di una circolazione relativamente limitata tra i Paesi all'interno della regione.

Le sequenze dei virus rabbia responsabili della recente epidemia nel Nord-Est (2008-2011) sono state inoltre analizzate. I risultati ottenuti indicano che i virus responsabili della recente epidemia di rabbia silvestre appartengono al lineaggio Europeo Occidentale del clade Cosmopolita. In particolare, è stato possibile individuare due sub-clades virali con due pattern geografici distinti. Entrambi i sub-clades presentano un'elevata omologia con i ceppi circolanti nei Balcani in particolare nelle aree confinanti dell'ex-Yugoslavia e le analisi evoluzionistiche effettuate sono fortemente indicative di una duplice introduzione degli stessi in un'area ed in un arco temporale ristretti.

SUMMARY

Rabies is an acute encephalomyelitis caused by a non-segmented negative strand of RNA virus belonging to the genus *Lyssavirus*, part of the *Rhabdoviridae* family. It is transmitted to humans by rabid animals, usually after a biting event. The virus affects the central nervous system, travelling from the point of infection to the brain via the nerve cells. Viral infection of the brain is almost invariably fatal from the moment when clinical symptoms appear, and can be classified in two forms: dumb rabies, which results in the paralysis of the victim, or furious rabies, characterised by aggression, excessive saliva production and acute respiratory distress.

Although international organizations have recognised the disease as being a high priority zoonosis, rabies is still widely neglected in most developing countries. The World Health Organization (WHO) annually encounters at least 10 million people receiving post-exposure treatment and 55,000 human deaths, mostly children in Asia and Africa (Knobel *et al.*, 2005). This estimation certainly needs a more accurate investigation, since modelling on human rabies in various enzootic countries revealed a clearly higher incidence of the disease in areas under survey (Ly *et al.*, 2009, Mallewa *et al.*, 2007).

Despite public health implications, rabies remains underestimated and diagnosed with difficulty in most endemic areas. Thus, the development of innovative diagnostic methods, as well as the study of the evolutionary dynamics of the viruses circulating in the reservoir species, is a crucial tool to both strengthen field surveillance and better plan the control programs in animals.

Two molecular tests (One-Step RT-PCR) for the diagnosis of rabies and further typing through pyrosequencing have been developed in this study. Pyrosequencing is an alternative to the Sanger method of sequencing, which relies on the detection of a released pyrophosphate molecule during the DNA synthesis (Ronaghi & Elahi, 2002). The protocols developed in this study target on two different genes, namely the genes encoding for the viral nucleoprotein (N) and for the viral glycoprotein (G). The first protocol (targeting the N gene) was mainly developed to rapidly identify a *Lyssavirus* responsible for the infection in field samples and is applicable to both human and animal specimens; the second protocol (targeting the G gene) was developed to differentiate a vaccine strain from a field (wild) rabies strain. The latter

protocol has also been applied to field samples collected from areas subject to oral vaccination of foxes with live attenuated vaccines during the Italian recent epidemic (2008-2011).

The molecular and evolutionary dynamics of rabies viruses circulating in West and Central Africa and more recently in North-eastern Italy have been investigated in this study. The phylogenetic analyses have been performed Maximum Likelihood methods (as implemented in PAUP* and PhyML) (Wilgenbusch & Swofford., 2003, Guindon & Gascuel., 2003). The evolutionary dynamics as well as the phylogeography of the obtained sequences have been inferred using the Bayesian Markov Chain Monte Carlo (MCMC) method available in the BEAST package (Drummond & Rambaut., 2007).

In a first study, viruses circulating in West and central Africa were analysed. Findings from this study confirm that a specific sub-lineage, called Africa 2, is circulating in the regions under survey. This sub-lineage was introduced approximately in 1845, most probably through the human-associated dispersal (i.e. the French colonization of the region). This study identified a strong geographical clustering of RABV sequences, which indicates a little transboundary spread of the infection.

A second study focused on viruses circulating in North-Eastern Italy. Findings from this study confirm that such strains fall into the Western European sublineage of the Cosmopolitan clade. Two genetic groups have been identified in Italy in the recent 2008-2011 epidemic, mainly distributed in two geographical areas. Viruses belonging to both clades have high similarity with those circulating in the Western Balkans, particularly Bosnia-Herzegovina, Croatia and Slovenia. Results from the evolutionary analyses strongly suggest the occurrence of two separate introductions in the same geographical area.

1 LITERATURE REVIEW

1.1 ETIOLOGY

Rabies virus (RABV) is the prototype species of the genus *Lyssavirus* in the family *Rhabdoviridae*, order *Mononegavirales*.

1.1.1 The family Rhabdoviridae

Rhabdoviruses infect a large diversity of organisms, including plants, insects, fish and mammals. They are bullet-shaped viruses approximately 75nm wide by 250nm long. A thin glycoprotein-studded coat encapsulates a nucleocapsid. The negative strand RNA is wrapped around a nucleoprotein (N) with a stoichiometry of 9 nucleotides to one N-monomer. This complex forms the template for the viral polymerase. When this complex is combined with the polymerase-phosphoprotein complex, the entire structure is referred to as the nucleocapsid. Most rhabdoviruses are transmitted by arthropods, and it has been postulated that *Lyssaviruses* were original rhabdoviruses that later spread to other various plant and animal hosts (Ammar *et al.*, 2009).

The *Rhabdoviridae* family is currently composed of six genera: *Vesiculovirus*, *Lyssavirus*, *Ephemerovirus*, *Novirhabdovirus*, *Cytorhabdovirus* and *Nucleorhabdovirus*. The vesiculoviruses, lyssaviruses and ephemeroviruses have been isolated from a variety of hosts and vectors, including mammals, fish and invertebrates. The remaining three rhabdoviruses have been shown to be more taxon-specific in their host preference, as i.e. *Novirhabdoviruses* infect fish, *cytorhabdoviruses* and *nucleorhabdoviruses* are borne by arthropods and infect various plants (Bourhy *et al.*, 2005).

1.1.2 The genus Lyssavirus

The genus *Lyssavirus* includes eleven species, among which the classical RABV is worldwide distributed and one of the main causes of human rabies deaths. Ten additional species, generally known as rabies-related lyssaviruses (RRVs), are currently recognised by the International Committee on the Taxonomy of Viruses (ICTV). It includes *Lagos bat virus* (LBV), *Mokola virus* (MOKV), *Duvenhage virus* (DUVV), *European bat lyssaviruses type 1* (EBLV-1) and *type 2* (EBLV-2), and *Australian bat lyssavirus* (ABLV). These species have established geographical niches, being distributed in bat populations worldwide, with the exception of MOKV, an African lyssavirus for which the reservoir species is still unresolved (Nel & Markotter., 2007). Except for LBV, spill-over transmission of RRV to humans has been

demonstrated, inducing a fatal encephalomyelitis clinically indistinguishable from the one caused by RABV (Nel & Markotter., 2007). Four new species have been recently classified in Aravan, Khujand, Irkut and West Caucasian bat viruses, all of them isolated from bats in Central Asia, with only one laboratory confirmed case of spill-over to humans (Leonova *et al.*, 2009). In addition, at least 4 new lyssaviruses have been described in bat reservoirs: a tentative species 8 lyssavirus (Delmas *et al.*, 2008), an unclassified lyssavirus, Shimoni bat virus, which seemed to be infecting a Commerson's leaf-nosed bat (*Hipposideros commersoni*) in Kenya (Kuzmin *et al.*, 2010) and the recently discovered Bakeloh Bat virus (BBV), isolated from an insectivorous bat in Germany (Freuling *et al.*, 2011). Lyssaviruses have been divided into two distinct phylogroups based on immunogenic and pathogenic properties of these viruses (Badrane *et al.*, 2001). The division was further supported by the phylogenetic analysis of the G coding region of these viruses (Johnson *et al.*, 2002). Phylogroup 1 contains species RABV, DUVV, EBLV-1, EBLV-2, ABLV and the newly discovered BBLV; phylogroup 2 contains MOKV and LBV. Sequencing data from other regions of the lyssavirus genome supports this division into phylogroups (Nadin-Davis *et al.*, 2002).

1.1.3 The rabies virus (RABV)

Rabies virus is the prototype virus of the *Lyssavirus* genus, and is associated with a variety of mammalian species worldwide. The main reservoir of RABV in Africa, Asia and Latin America is the domestic dog, while the racoon dog and skunks and the red fox are the important reservoirs in the Americas and Europe, respectively. In addition to terrestrial mammals, insectivorous bats act as reservoirs of rabies in North America, and hematophagous bats are reservoirs in South America.

There is strong evidence to suggest that bat lyssaviruses predate terrestrial carnivores, including two ancient spills-over with RABV (Badrane & Tordo., 2001) and it has recently suggested (Bourhy *et al.*, 2008) that terrestrial rabies originated from domestic dogs in the south of the Indian subcontinent @1500 years ago. The first spillover is thought to have occurred in North America from bats to racoons, the second occurred in an unknown region leading to terrestrial rabies virus lineage found worldwide (Badrane & Tordo., 2001). Modern examples of spills-over events are i.e. the identification of a bat-associated virus in skunks in the USA, reported in 2006 (Leslie *et al.*, 2006) and EBLV-1 infection in terrestrial mammals in continental Europe, such as in cats (Dacheux *et al.*, 2009) and stone martens (Muller *et al.*, 2004).

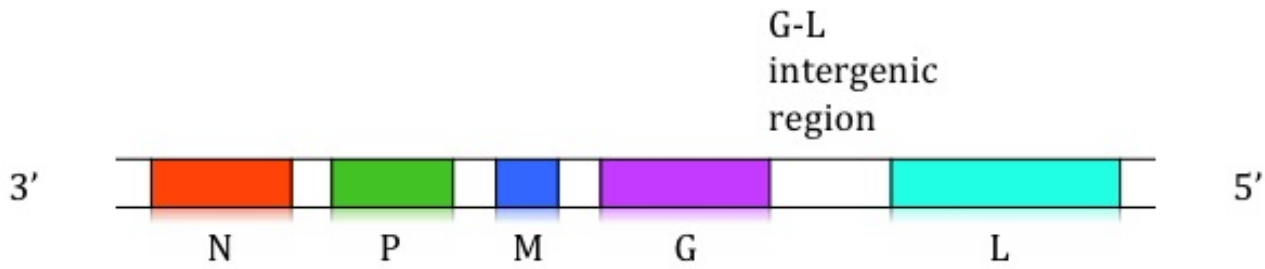
1.1.4 The genes and proteins of the rabies virus

All Lyssaviruses share many biologic and physicochemical features as well as amino acid sequence characteristics that classify them with the other rhabdoviruses. These include the bullet-shaped morphology, the helical nucleocapsid (NC), the structure of the RNA genome, and the organization of the structural proteins. The five structural proteins of the virion include a nucleocapsid protein (N), phosphoprotein (P, M1 or NS), matrix protein (M or M2), glycoprotein (G), and RNA-dependent RNA polymerase or large protein (L). The genomic RNA, as for all the viruses with negative-polarity genome, is not infectious and it cannot be translated directly into protein. The first event in infection is transcription of the genome RNA to produce complementary monocistronic messenger RNAs (mRNA) from each of the viral genes or cistrons in the genome. The viral proteins are synthesised from the monocistronic mRNAs. (Jackson & Wunner., 2002)

The RABV genome is composed of @ 12 000 nucleotides and, as for the remaining lyssaviruses, it codes for five genes. At the 3' end of the nucleotide genome of RABV is a non coding (leader) Le sequence. Downstream the Le sequence, in sequential order, the five structural genes (N, P, M, G and L) and a non coding trailer (Tr) sequence follow (see figure 1). The genes are separated by short sequences that represent the intergenic regions of the genome. These short intergenic regions are located between the N and P genes and between the P and M genes and the M and G genes. The remaining intergenic region between the G and L genes contains a long stretch of 423 nucleotides in the genome. The G-L intergenic region is sufficiently long to represent a potential gene but lacks an ORF for a detectable protein. It has been the designation of remnant gene or pseudogene (Ψ), recognizing it once represented an ORF of sufficient size to code for a recoverable protein (Tordo *et al.*, 1986).

Within the genus *Lyssavirus*, the genes have different levels of conservation, which would impact the choice of gene used for various studies. With regards to the relative conservation of these genes, the N-gene is the most conserved, followed by the L, M, G and P genes (Wu *et al.*, 2007).

Figure 1. The rabies virus genome depicting the genes as well as intergenic regions.



1.1.4.1 The nucleoprotein

The nucleoprotein gene is the most conserved of all the rabies virus genes (Kissi *et al.*, 1995) and thus is frequently used in phylogenetic and molecular epidemiological studies.

The nucleoprotein is a phosphorylated protein consisting of 450 amino acids. As mentioned previously, it binds to the viral RNA during virion formation to form complex which protects the RNA from degradation during morphogenesis, and forms an integral part of the nucleocapsid in the mature virion. The nucleoprotein is also needed for the binding of the polymerase complex during transcription (Schoehn *et al.*, 2001).

The nucleoprotein also plays a role in the generation of immune response. Vaccines that are constructed of the nucleoprotein were shown to provide protection from peripheral challenges from rabies virus in experimental conditions (Fekadu *et al.*, 1991). Epitopes of the N are recognised by B and T helper cells (Goto *et al.*, 2000). However, despite the evidence that N has an immunogenic effect, speculation continues about its importance in producing protective immune responses (Drings *et al.*, 1999).

The nucleoprotein of rabies has also been shown to be a superantigen (Lafon & Galelli, 1996). It shuts off rabies-specific immune response while the virus is travelling through the nervous system. Superantigens bind to class II major histocompatibility complex molecules that are expressed on antigen presenting cells, while simultaneously binding to the variable region of T-cell receptor β chains. This results in stimulation of the T-cells expressing the correct β chain element, leading to cytokine release and systemic shock, allowing the virus particles to escape rabies-specific immune response (Fraser *et al.*, 2000).

1.1.4.2 The phosphoprotein

The phosphoprotein forms the non-catalytic part of the polymerase complex that transcribes and translates the genetic material of the rabies virus. The phosphoprotein gene has been used to elucidate the phylogenetic relationships between the viruses of the lyssavirus genus (Nadin-Davis *et al.*, 2002). The phosphoprotein is 297 amino acids in length and has a variety of phosphorylated forms (Jackson & Wunner., 2002) (Gupta *et al.*, 2000). The phosphoprotein mediates the connection between the nucleoprotein-RNA complex and the RNA polymerase (Chenik *et al.*, 1994). The phosphoprotein also binds to free nucleoprotein, thereby preventing the nucleoprotein from aggregating in the cytoplasm and being unavailable for encapsidation (Liu *et al.*, 2004).

1.1.4.3 The matrix protein

The matrix protein is the smallest of the virion proteins, consisting of 202 amino acids (Jackson & Wunner., 2002). It is a multifunctional protein that plays a crucial role in virus assembly and budding, through the condensation of the RNP complex into a “skeleton form” and also assists in virus association with the plasma membrane (Mebatsion *et al.*, 1999). It also inhibits transcription of the RNP by binding closely to it, and stimulates replication of the virus (Finke *et al.*, 2003).

The matrix protein has been shown to self-associate via a string of amino acids in the n terminus that binds to a region on the globular domain, resulting in an enhanced affinity for membranes. This interaction differs between matrix proteins from vesiculoviruses and lyssaviruses, and may help explain the different cytopathic effects of these viral genera (Graham *et al.*, 2008).

The matrix protein also plays a crucial role in apoptosis as it interacts directly with the cytochrome C oxidase subunit of the mitochondrial respiratory chain and interferes with the activity of this subunit which induces programmed cell death (Gholami *et al.*, 2008).

1.1.4.4 The glycoprotein

The glycoprotein of rabies virus forms structures on the surface of the virus. It is 505 amino acids in length despite the G-coding region coding for 524 amino acids. These first 19 amino acids represent the signal peptide that gives the membrane insertion signal and allows entrance of the virus into the cell (Jackson & Wunner., 2002). The glycoprotein forms surface spikes that are responsible for the generation of immune response and binding of virus

neutralising antibodies. For this reason, it has been widely studied for vaccine formulation and development of biological (Artois *et al.*, 1990, Drings *et al.*, 1999, Kieny *et al.*, 1984). Aggregation of the glycoprotein at the cytoplasmic membrane of human nerve cells also functions as trigger of apoptosis (Prehaud *et al.*, 2003).

1.1.4.5 The RNA polymerase

The RNA polymerase is the largest protein in the virion, composed of 2142 amino acids in the Pasteur virus strain. The RNA polymerase gene has been used in phylogenetic studies to determine the genetic relationships among rhabdoviruses (Bourhy *et al.*, 2005). The number of amino acids composing the RNA polymerase differs between different strains, with the SADB19 strain RNA polymerase being composed of 2127 aa (Jackson & Wunner., 2002). The polymerase binds to the RNA complex via the phosphoprotein to form a complex, which results in the replication and transcription of viral RNA (Chenik *et al.*, 1994). The RNA polymerase also forms an important role in the start of infection by initiating the primary transcription of genomic RNA once the nucleocapsid is released into the cytoplasm of the infected cell (Jackson & Wunner., 2002).

1.1.4.6 G-L intergenic region

The G-L intergenic region is a large non-coding region of 423 nucleotides, located between the G and L coding regions. The discovery of start and stop consensus sequences at the ends of the intergenic region has led to conclude that it may be a remnant gene (Tordo *et al.*, 1986). This possibility was supported by the fact that a related fish rhabdovirus (infectious hematopoietic necrosis virus, IHNV) contains a sixth gene similar to the length of the G-L intergenic region (Kurath & Leong., 1985). However, all RABVs contain only the downstream motif, but only one lineage of laboratory strains was proven to contain both upstream and downstream signals (Ravkov *et al.*, 1995).

The region has been extensively used in phylogenetic studies to discriminate between closely related isolates of RABV, as it is not a highly conserved region, due to the lack of coding for a protein.

1.2 RABIES PATHOGENESIS

Rabies virus enters the body through either direct contact with mucosal surfaces or, more commonly, wounds made by the bite or scratch of a rabid animal. In very rare cases, rabies has been associated to handling of infected carcasses that were prepared for human consumption. However, in this instance, meat consumption did not proved to be the cause of the disease, as the meat was shared among people who did not develop rabies_([Wertheim *et al.*, 2009).

The virus then replicates in non-nervous tissues, or enters nerve cell and begins replication in these celles (Mrak & Young., 1994). The glycoprotein acts as the receptor and facilitates uptake into nerve cells through membrane fusion (Gaudin., 2000). The virus then travels via retrograde axoplasmic flow to the Central nervous system. It has been suggested that since the phosphoprotein binds to the dynein light chain 8 (Jacob *et al.*, 2000), dynein motor complex-mediated transport of virus RNPs along microtubules is responsible for the movement of the virus in the Central nervous system. However, this finding (Mebatsion., 2001) showed in 2001 that in cell culture system the replication of light chain binding defective rabies virus mutants was indistinguishable from wild type replication. The proximity of the site of virus entry to the Central nervous system and the severity of wound generally shorten the incubation period (Mrak & Young., 1994).

After inoculation of the Central nervous system the virus moves into the brain as well as other peripheral non-nervous tissues such as the salivary glands. The virus becomes widely spread throughout the body by the time that clinical symptoms manifest (Mrak & Young., 1994).

1.3 SIGNS AND SYMPTOMS

1.3.1 Symptoms in animals

There are no known definitive or species-specific clinical signs of rabies beyond acute behavioural alterations. Severity and variation of signs may be related to the specific site of primary lesion or to the viral dose, and route of infection. The prodromal period usually follows a bite by several weeks. However, periods less than 10 days to several months are well documented. Initial signs of rabies are nonspecific and may include anorexia, lethargy, fever, dysphagia, vomiting, stranguria, straining to defecate and diarrhoea. A gradual and subtle alteration in behaviour, such as increased aggressiveness or unusual docility, and a qualitative change in phonation, as well as increased vocalisation, may be noted fairly early in the clinical course. Cranial nerve manifestations may include facial asymmetry, trismus, choking, a lolling tongue, drooling, drooping of the lower jaw, prolapse of the third eyelid, and anisocoria (Jackson & Wunner., 2002).

An acute neurological period usually follows, characterised by a generalise increase in neurologic activities, associated to hyperesthesia to auditory, visual, or tactile stimuli and sudden and seemingly unprovoked agitation and extreme aggressive behaviour toward animate or inanimate objects. Wild animals may lose their apparent wariness to human. The disease culminates in coma and generalised multiorgan failure with death in 1-10 days after primary signs (Jackson & Wunner., 2002).

1.3.2 Symptoms in humans

Rabies in humans has a general incubation time of 20 to 60 days, however, there have been reports of rabies becoming symptomatic after approximately 5 days though the incubation period can last as long as 16 months from the initial exposure (Mrak & Young., 1994). First symptoms are generally non-specific and consist of general malaise, fever, anxiety. Tingling at the area of the bite may occur and is related to ganglioneuritis. After a couple of days (2 to 10 days), neurological signs manifest, either as encephalic or paralytic form. In encephalitis rabies, symptoms range from the classic hydrophobia and salivation to aerophobia, confusion and convulsions. These symptoms are episodic and between them, the patient is usually lucid. Eventually paralysis and coma develop, leading to patient death (Jackson & Wunner., 2002).

In paralytic rabies, descending weakness of the extremities without the loss of consciousness is the primary symptom. This weakness of the limbs begins at the site of the bite and soon

spreads to other limbs. Eventually, the paralysis spreads to the respiratory systems and the patient usually dies due to asphyxiation. The precise mechanism through which the alternative presentations are triggered is not known, with the same virus isolates inducing either encephalic or paralytic manifestations (Hemachudha *et al.*, 2003).

1.4 EPIDEMIOLOGY

Rabies viruses are adapted to various animal species (acting as reservoir) on which they depend for their existence. Rabies virus perpetuation involves introduction into a susceptible host, most typically by transmission via an animal of the same species, and then the infection, dissemination, and multiplication of the virus within the host (Jackson & Wunner., 2002).

Although most mammals are susceptible to infection by any lyssavirus, each viral strain is associated with and maintained by a reservoir host. Viral infection of a species that does not normally maintain that virus, referred to as a spill-over infection, occurs frequently but rarely resulting in spread within the new host (Nadin-Davis & Real., 2011). As for instance, although rabies is a zoonotic disease, human infections and deaths are an unfortunate consequence of biologic processes of virus maintenance in which humans play no significant role.

Almost invariably, the route by which RABV is introduced into a new host is direct, and the most important mode of transmission is by inoculation by bite of infectious virus present in saliva. The process by which RABV circulates within diverse species of mammals causing serial infections is referred as the maintenance of transmission cycle of the virus. Since humans are rarely the source of virus for subsequent human infections, humans do not contribute to the maintenance of RABV. The reservoir for rabies is the animal pool that circulates RABV, with only occasion spills-over to humans (Jackson & Wunner., 2002).

1.4.1 Rabies phylogeography

As a result of the viral host adaptation, physical landscape features limiting spatial spread of a reservoir host will invariably limit the spread of any virus that it harbours (Nadin-Davis & Real., 2011). The only means by which the virus can overcome this limitation is to adapt to a new host. As a consequence of this epidemiological feature, viral population will exhibit pronounced phylogeographical patterns (Nadin-Davis & Real., 2011).

With regards to the classical rabies virus (species RABV), a clear picture of the extent of viral worldwide diversity and phylogeny has been drawn (see Figure 2) (Badrane & Tordo., 2001, Bourhy *et al.*, 2008, Kissi *et al.*, 1995, Nadin-Davis *et al.*, 2002) (reviewed in (Nadin-Davis & Real., 2011)).

Recently, the application of ML and Bayesian coalescent approaches to analyse lyssavirus sequence data provides robust methods for estimating nucleotide substitution rates (number of nucleotide substitution/site/year). This value has been estimated for RABV-coding regions

as ranging from 1×10^{-4} to 4×10^{-4} subs/site/year with no clear variation according to the reservoir. Rates for the non-coding intergenic region G-L tend to be higher (0.777×10^{-3} to 1.676×10^{-3} subs/site/year), probably due to the relaxed constraints on this segment of the genome (reviewed in (Nadin-Davis & Real., 2011)).

Based on the same approach, the time of most common recent ancestor (TMRCA) for most of RABVs lineage has been estimated. Estimated TMRCA for RABVs circulating in terrestrial mammals dated back 749 years ago using the N gene sequences available (with 95% highest posterior density (HPD) range from 363-1215 years) and 583 using the G gene sequences (with 95% HPD range from 222-1222 years) (Bourhy *et al.*, 2008). Similarly, TMRCA for RABVs circulating in flying mammals in the Americas dated back 761 years ago using the N gene sequences available (with 95% HPD range from 373-1222 years) (Bourhy *et al.*, 2008). Based on those findings, the two distinct branches of RABVs that circulate in American bats and terrestrial mammals emerged approximately at the same time (Bourhy *et al.*, 2008).

1.4.2 RABV clades

The Indian clade has the most basal position of all RABVs associated with terrestrial mammals. It is maintained in nature by the dog (Nanayakkara *et al.*, 2003). It has been proposed that the ancestor of this clade may have been the progenitor for all RABVs lineages harboured by terrestrial mammals (Bourhy *et al.*, 2008).

The Asian clade is a heterogeneous lineage, subdivided in multiple regional subgroups, for which the dog is the principal reservoir (Bourhy *et al.*, 2008). This clade likely spread from China to neighbouring countries, including islands, such i.e. Philippines and Indonesia (Gong *et al.*, 2010, Nishizono *et al.*, 2002) (Susetya *et al.*, 2008).

The Cosmopolitan clade includes the genetic group previously referred to as Africa 1 (Bourhy *et al.*, 1993, Kissi *et al.*, 1995), have been worldwide distributed mainly throughout human-assisted movement of pets from Europe during the colonialism age. Therefore, this clade includes viruses collected from several areas of the Americas and the Caribbean, other than Europe, the Middle East and Africa, encountering for different host reservoirs, according to the geographical location and ecological situation (Bourhy *et al.*, 2008, Smith *et al.*, 1992).

Viruses from the Africa 2 clade are distributed in West and Central Africa, with the host reservoir the dog (Bourhy *et al.*, 1993, Kissi *et al.*, 1995). Chapters 3.1 and 3.2 of this study will describe the evolutionary and phylogenetic analyses of those viruses.

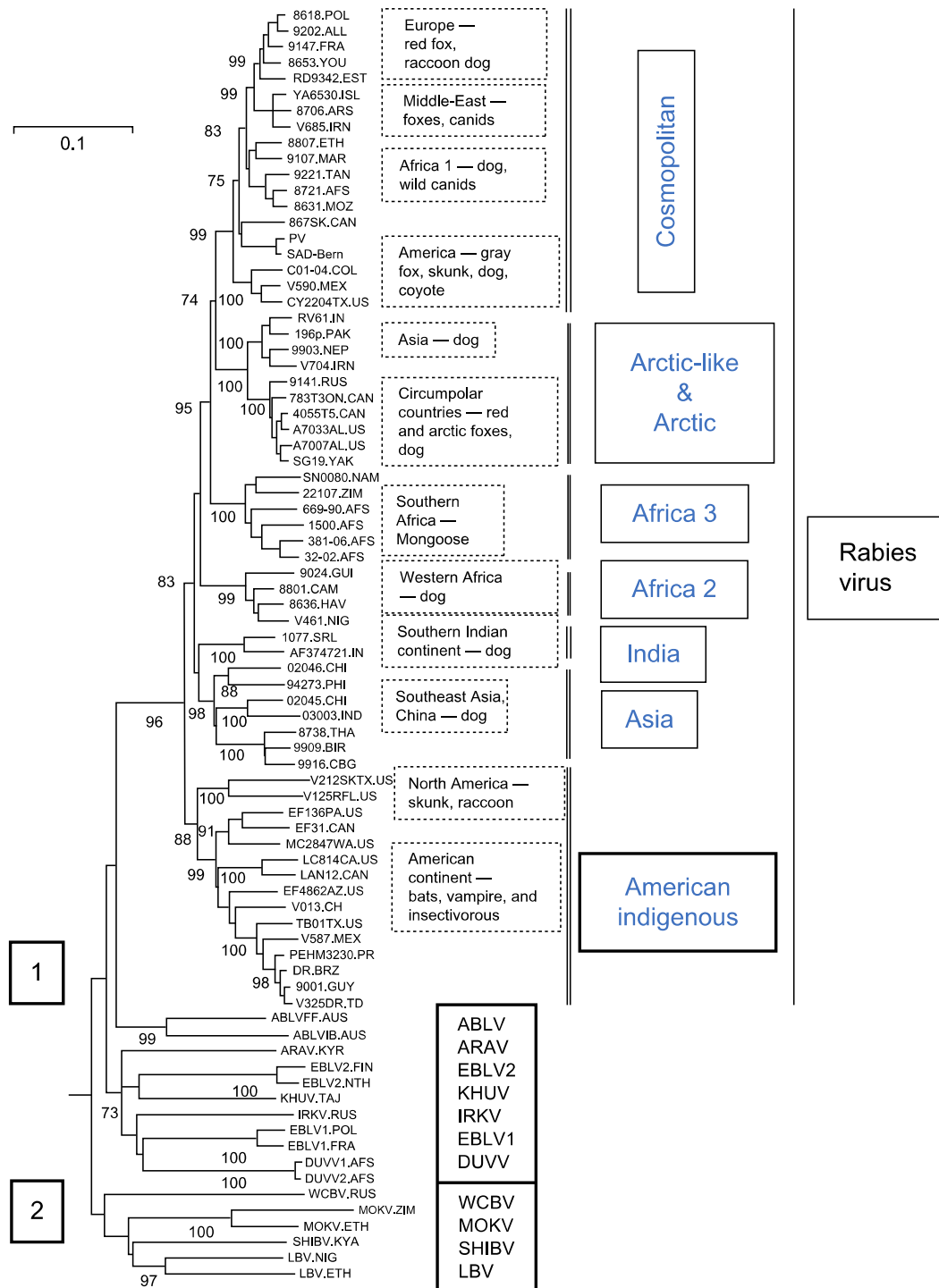
The Africa 3 clade comprises viruses well adapted to the yellow mongoose in Sub-Saharan Africa (Bourhy *et al.*, 1993, Kissi *et al.*, 1995). Molecular studies have not been able to depict a clear picture on the emergence and evolution of these viruses. A comparison on the population dynamics between the viral population of canid and mongoose lineages suggests the latter was the most ancient RABV in the region (Van Zyl *et al.*, 2010).

Viruses from a same clade, called Arctic-related or Arctic-like, have been characterised from a wide area of Asia and in northern climates areas (Mansfield *et al.*, 2006, Nadin-Davis *et al.*, 2007) (Nadin-Davis *et al.*, 1993). Phylogenetic analysis suggests that Indian isolates fall into a basal position, thus suggesting that the entire clade evolved from Indian dog viruses, with subsequent differentiation into genetic subclades according to the geographical location and transmitted by different host species (Kuzmin *et al.*, 2008, Nadin-Davis *et al.*, 2007).

A group of virus genetically related and circulating in several mammalian species in the Americas are classified as American indigenous clade, also referred to as American bat (Nadin-Davis & Real, 2011). This group includes viruses adapted to terrestrial species other than flying mammals and the common origin from a bat-associated ancestor has been hypothesised and strengthened by the occurrence of a species jump event (Leslie *et al.*, 2006). Viruses belonging to the same lineage have been associated to different insectivorous bats in Northern America and to vampire bats (*Desmodus rotundus*) in Latin America (reviewed in (Nadin-Davis & Real, 2011)).

With the exception of the American indigenous clade, most of RABV clades are associated totally or in part to the dog reservoir. Particularly for Cosmopolitan and Arctic-like clades, other Canidae species are involved in the maintenance of RABV in nature, such as foxes, skunks, mongoose, jackals and wolves). This finding suggests that the emergence of RABV progenitor was closely related to the dog reservoir and that in rare occasion spills-over resulted in a self-perpetuation of the viral infection in a new host (Bourhy *et al.*, 1999, Johnson *et al.*, 2003, Paez *et al.*, 2005, Sabeta *et al.*, 2007).

Figure 2. Phylogenetic tree of 80 RABVs representative of known lyssavirus diversity (from (Nadin-Davis & Real., 2011)), performed using the neighbour-joining method as implemented by the MEGA 4 program (Tamura *et al.*, 2007).



1.5 DIAGNOSIS

1.5.1 Recommended classic diagnostic techniques

Validated diagnostic have been widely used to implement rabies control strategies worldwide (Anonymous , 2005,Anonymous , 2010). The standard methods have provided accurate information of animal and human rabies cases thus supporting surveillance for this disease.

Moreover, conventional diagnostic tests for rabies are not labour intensive and rely upon low throughput. For numerous laboratories in the developing world, cost and simplicity are critical factors for the disease diagnosis and therefore classical methods are still strongly recommended cby the international organizations (Anonymous , 2005,Anonymous , 2010).

1.5.1.1 Fluorescent antibody test

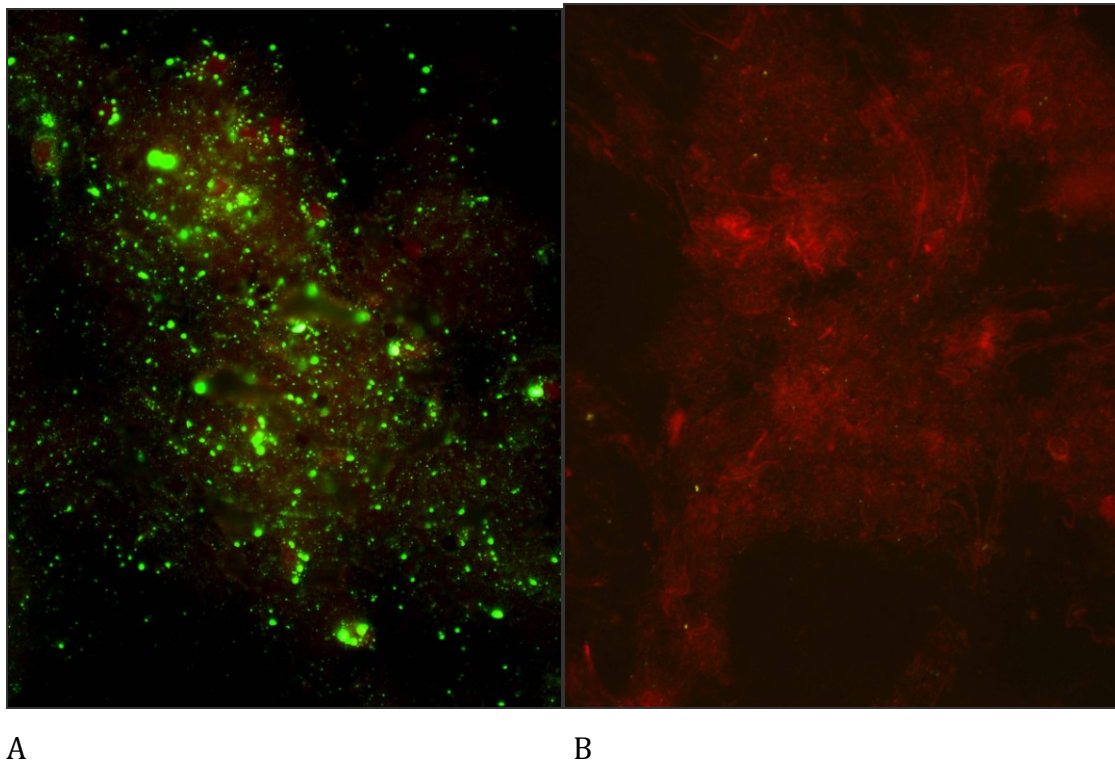
The Fluorescent Antibody test (FAT) (Anonymous , 2005,Anonymous , 2010) is universally considered the gold standard for rabies diagnosis. The test is mainly a *post-mortem* diagnostic tool since it is performed on brain specimens and not applicable to other kinds of samples (i.e., cerebrospinal fluid and saliva) and only implemented with difficulty to skin biopsies (Crepin *et al.*, 1998). Its sensitivity is strongly affected by the freshness of the sample and by the viral strain (Kamolvarin *et al.*, 1993,Vazquez-Moron *et al.*, 2006). As a matter of fact, commercially available reagents for rabies diagnosis are designed for the detection of classical rabies virus (RABV). Data emerged from European interlaboratory trials results, confirms that the FAT technique represents a good compromise with both satisfactory sensitivity and specificity, as only a few false positive (from 1.6% to 5.8%) and false negative results (1.6%) were detected (Robardet *et al.*, 2011). The FAT can be completed in less than two hours. The good performances of this test make it the current test of choice for post mortem diagnosis, notably for veterinary laboratories.

The method relies on the ability of a detector molecule (usually fluorescein isothiocyanate) conjugated to a rabies specific antibody. This complex is able to bind a rabies antigen and the binding complex is visualised using fluorescent microscopy (see figure 3). Anti-rabies monoclonal as well as polyclonal antibodies, selected for the test are generally specific for the nucleoprotein, a viral protein produced in abundance during productive infection (Jackson & Wunner., 2002). The sample is directly analysed by using a microscopic test (FAT), thus allowing for the identification of rabies virus-specific antigen (Anonymous , 2005,Anonymous , 2010).

Figure 3. Fluorescent antibody test (FAT) on fresh touch impression (200X magnification).

A: positive: apple-green fluorescence detection of rabies virus nucleoprotein. B: negative.

Red background: Evans blue counterstain.



1.5.1.2 Mouse Inoculation Test and Rabies Tissue Culture Infection Test

The rationale for the use of virus isolation from a sample where there is a suspicion of infection with rabies virus is always recommended (Anonymous , 2005,Anonymous , 2010).

Two methods are currently implemented worldwide for virus isolation, namely the Mouse Inoculation Test (MIT) and the Rabies Tissue Culture Infection Test (RTCIT). Both methods are based on the propagation and isolation of the virus either *in vivo* or in a cell-based system (Anonymous , 2005,Anonymous , 2010). Both methods require a longer turnaround times than the FAT, with 4 days and 4 weeks needed for RTCIT and MIT, respectively.

Data from European interlaboratory trials results, reveals that the MIT technique is associated with the occurrence of a relatively high number of false negative results if compared to both standard and molecular techniques (Robardet *et al.*, 2011). Thus, as recommended by the international organisations, the use of the MIT should be abandoned in favour of the more rapid and sensitive RTCIT (Anonymous , 2005,Anonymous , 2010).

1.5.2 Other recommended methods

1.5.2.1 ELISA-based methods

ELISA techniques can be used for the detection of rabies antigens. These tests are rapid, cheap, easy to use, and safe because they do not require the use of infectious virus. Although potentially suitable for diagnostic purposes, these methods are still not used on a large-scale. A validated ELISA for the rapid detection of antigen in brain samples has been evaluated under field conditions in different countries (Bourhy *et al.*, 1989) (Jayakumar *et al.*, 1989, Morvan *et al.*, 1990, Saxena *et al.*, 1989) and the international organisations recommend its use (Anonymous, 2005, Anonymous, 2010). Another reliable, rapid, and transferable ELISA method named WELYSSA is designed to detect lyssaviruses belonging to all seven genotypes circulating in Europe, Africa, Asia, and Oceania (Xu *et al.*, 2007).

1.5.3 Methods awaiting further standardisation

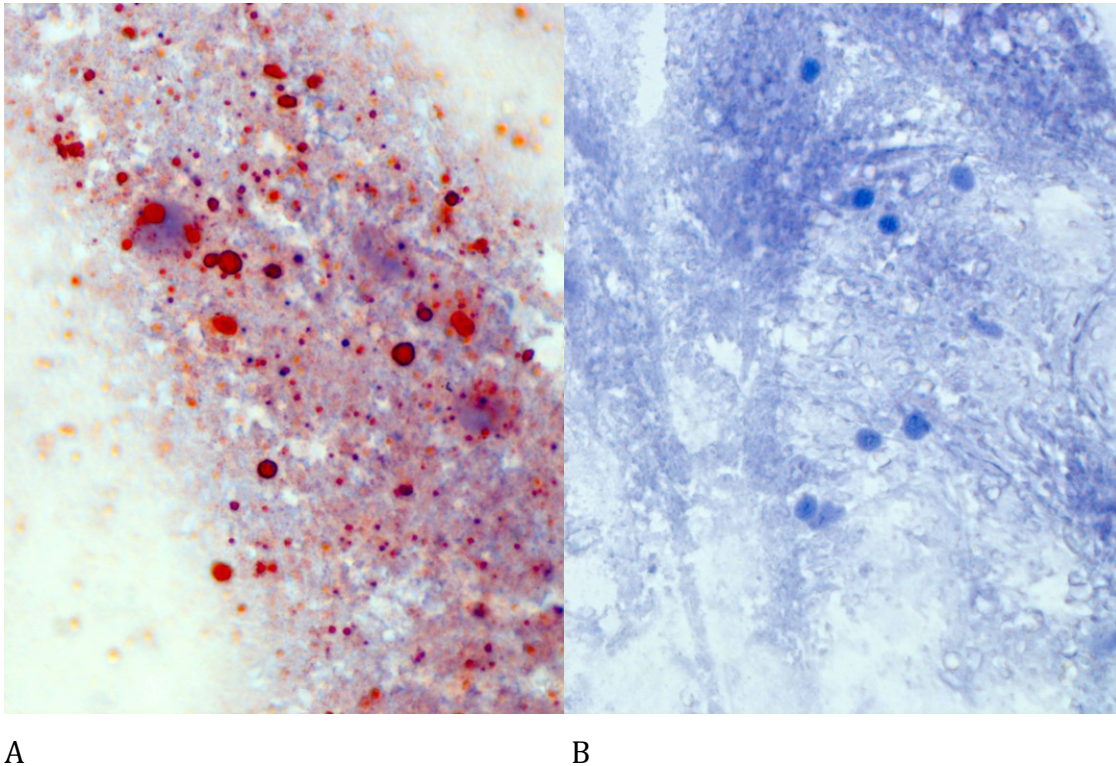
1.5.3.1 Direct Rapid Immunohistochemical Test

A direct Rapid Immunohistochemical Test (dRIT) for the postmortem detection of rabies virus antigen in brain smears has been recently developed. Using a cocktail of highly concentrated and purified biotinylated monoclonal antibodies, rabies antigen can be detected by direct staining of fresh brain impressions within 1 hour (see figure 4).

Performed on brain tissues, dRIT has proven as sensitive as the FAT for fresh specimens, since they both rely on antibodies specifically detecting the same viral protein. The dRIT antibody cocktail is biotinylated such that, following a short incubation with a streptavidin-peroxidase complex, antibody-antigen binding complexes can be visualised through the addition of the substrate, 3-amino-9-ethylcarbazol (Lembo *et al.*, 2006).

The purpose for development and standardisation of dRIT is to offer an alternative diagnostic test that would enable developing countries to perform routine rabies surveillance (Durr *et al.*, 2008, Lembo *et al.*, 2006). However, it must be underlined that this method still requires a basic equipment and, most important, a well-experienced laboratorist for result interpretations.

Figure 4. Direct rapid immunohistochemistry test (DRIT) on fresh brain touch impressions (400X magnification). A: positive: red specific staining identification of rabies virus nucleoprotein. B: negative. Blue background: hematoxylin counterstain.



1.5.3.2 Immunochromatographic techniques

Another method for the detection of rabies virus antigen from postmortem samples is a recently developed rapid immunodiagnostic test (RIDT) based on the principles of immunochromatography (Kang *et al.*, 2007).

Preliminary studies with a limited number of samples showed that the RIDT might have great potential as a useful method for rabies diagnosis without the need for laboratory equipment ((Markotter *et al.*, 2009, Servat *et al.*, 2012). However, kit standardisation, improvement of sensitivity and a final validation using a wider panel of field samples are still needed, before this test could be relied upon and be used as an alternative for the gold standard FAT.

1.5.3.3 Molecular-based methods

Molecular methods proved to be a sensitive and specific tool for routine diagnostic purposes in animals, particularly in decomposed samples or archival specimens (Araujo *et al.*, 2008, Biswal *et al.*, 2007, David *et al.*, 2002, Kulonen *et al.*, 1999) and an essential tool for intra-vitam human diagnosis (Crepin *et al.*, 1998, Dacheux *et al.*, 2008).

Generally speaking, the lack of standardization and inter-laboratory reproducibility and quality issues such as cross-contamination events or false negative results, have been recognised as major constraints to the use of molecular methods. International organizations are therefore extremely cautious in suggesting standard molecular protocols for rabies diagnosis. OIE and WHO recommend to apply RT-PCR as typing method only in specialised laboratories, following however recommended validation guidelines (Anonymous , 2005, Anonymous , 2009), and not as routine techniques for *post mortem* diagnosis. From a more comprehensive point of view, a diagnostic approach based on the application of good laboratory practices, inter-laboratory standardisation and the complementary use of both classical and molecular protocols will help overcome the intrinsic limitations of diagnostic methods currently available for rabies (Dacheux *et al.*, 2010).

1.5.3.4 Internal Amplification Controls

The use of an internal amplification control (IAC) is recommended to eventually identify false negative samples resulting from the presence of PCR inhibitors or degradation of the nucleic acid (Hoorfar *et al.*, 2004). In this regard, endogenous (i.e. a housekeeping gene) or exogenous nucleic acid (i.e. a microorganisms which is usually not found in the sample type to be tested) can be used as internal control. Such internal controls can be applied to many different tests, running independently of the specific virus assay and currently available in molecular diagnostic laboratories. Alternatively, a competitive IAC (i.e. the target and the IAC are amplified with one common set of primers and under the same conditions in the same PCR tube) can be developed bearing in mind that all these approaches have both advantages and limitations. For example, if a competitive IAC approach is applied, the different amplification assays will be competing for the same reagents in the same tube, thus target detection sensitivity may be adversely affected (Hoorfar *et al.*, 2004) (EPA., 2004).

1.5.3.5 Reverse-transcriptase polymerase chain reaction (RT-PCR)

Various conventional RT-PCR protocols for the diagnostic amplification of lyssavirus genome fragments have been published (Crepin *et al.*, 1998, Dacheux *et al.*, 2008, David *et al.*, 2002, Gupta *et al.*, 2001, Kulonen *et al.*, 1999, Whitby *et al.*, 1997). Since primers were selected from conserved regions of the genome, most assays amplify parts of the N gene as earlier proposed. However, another region of the lyssavirus viral genome, the RNA polymerase gene,

can also be used. This gene encompasses several nucleotide blocks (such as block III) that have been highly conserved during lyssavirus evolution (Bourhy *et al.*, 2005).

In generic approaches intended to detect all lyssaviruses either hemi-nested or fully nested amplifications are used and have applications for both antemortem (saliva, CSF, brain) and postmortem samples (principally brain tissue). Some of these diagnostic procedures are also applied for further virus characterization, including sequencing reaction or restriction fragment length polymorphism (RFLP) (Nadin-Davis *et al.*, 1993). Also, strain-specific RT-PCRs have been developed to distinguish various rabies virus (RABV) strains in a particular region.

1.5.3.6 Real time RT-PCR

Various PCR assays using TaqMan technology have been described (Hoffmann *et al.*, 2010, Hughes *et al.*, 2004, Wacharapluesadee *et al.*, 2008, Wakeley *et al.*, 2006). As for conventional PCR methods, PCR assays using TaqMan technology have applications for antemortem and postmortem samples (Hemachudha & Wacharapluesadee, 2004, Wacharapluesadee & Hemachudha, 2010).

It should be pointed out that probe-based methods are prone to detection failures due to single mutations of strains under examination (Hoffmann *et al.*, 2010, Hughes *et al.*, 2004). Thus the genetic diversity among lyssaviruses may hamper the use of a single assay for all lyssaviruses. To overcome this issue, assays relying on the use of a DNA intercalated molecule (as i.e. SIBR green) can be used in alternative to avoid the occurrence of false negative results (Hayman *et al.*, 2011a). In such cases however, the occurrence of false positive results should be seriously taken into account. As for gel based PCRs, endogenous gene fragments may represent the amplified products.

1.5.3.7 Nucleic acid sequence-based amplification (NASBA)

The NASBA technique involves the use of three enzymes (reverse transcriptase, RNase H and T7 RNA polymerase) to synthesise multiple copies of target RNA under isothermal conditions. The amplified RNA is detected using an automated reader, enabling rapid throughput testing. As for other infectious diseases, the use of the NASBA technique has been developed and applied also to rabies diagnosis (Sugiyama *et al.*, 2003) (Wacharapluesadee & Hemachudha, 2001).

1.5.3.8 Loop-Mediated Isothermal Amplification (LAMP)

Using the LAMP technique, amplification is achieved through the specific binding of two inner and two outer primers to the target sequence. The inner primers initiate strand synthesis whilst the outer primers displace the inner primers, allowing them to self-anneal to the nascent strand. This creates hairpin structures that trigger further strand synthesis that in turn lead to concatenation of the target sequence (Notomi *et al.*, 2000) (Nagamine *et al.*, 2002).

As for other molecular techniques, the application of RT-LAMP assays in field conditions is challenged by the genome diversity of lyssaviruses (Boldbaatar *et al.*, 2009, Hayman *et al.*, 2011b, Saitou *et al.*, 2010).

1.5.3.9 Microarray detection of lyssaviruses

Microarray linked to sequence independent PCR amplification offers the ability to rapidly identify pathogenic viruses from post-mortem samples.

A preliminary study has demonstrated the ability of a microarray to detect known lyssavirus species (Gurralla *et al.*, 2009). In addition, a research has been undertaken to use microarray technique for *Rhabdoviridae* genetic classification (Dacheux *et al.*, 2010). However, the applicability of this technique to large-scale surveillance is still to be demonstrated.

1.6 FUNDAMENTALS OF APPLIED EXPERIMENTAL TECHNIQUES

1.6.1 Sequencing

Sequencing allows the nucleotide sequence of a portion of DNA to be determined. Identifying the sequence of nucleotides allows scientists to determine the amino acid sequence proteins codified by a particular segment of genome. Sequences can be compared, resulting in the determination of phylogenetic relationships between various selected organisms.

1.6.1.1 Sanger sequencing

To date, Sanger sequencing (or classical sequencing) is the most applied method for genome sequencing. Since 1977, many improvements have been made to the Sanger method of sequencing (Sanger *et al.*, 1992), the most notable being the introduction of fluorescent labelled dideoxy nucleotides with different colours which allows the four reactions of the Sanger method to be done in one reaction. After the chain elongation, the reaction is run through a machine that detects the fluorescence signal values of the various fragments and generates the DNA sequence from this information (Sambrook & Russel., 2001).

1.6.1.2 Pyrosequencing

Pyrosequencing is an alternative to the Sanger method of sequencing, which relies on the detection of a released pyrophosphate molecule during the DNA synthesis (Ronaghi & Elahi., 2002). The enzymatic cascade that generates visible light (which is proportional to the number of incorporated nucleotides) begins when inorganic pyrophosphate is released, as a result of nucleotide incorporation by DNA polymerase. The released pyrophosphate then is converted to ATP, which is used to oxidise luciferin. Because the added nucleotide is known, the sequence of the template can be determined (Ronaghi & Elahi., 2002). This method can be used for both single-base sequencing and whole-genome sequencing, depending on the format used (Nyren., 2007).

1.6.2 Phylogenetic techniques

Phylogenetic trees have been used to represent the historical relationships of groups of organisms. Until recently, those relationships were basically based on antigenic (morphological) characteristics. With the advent of molecular sequencing, extensive new data set is currently available and updated.

There are two primary approaches to tree construction: algorithmic and tree-searching. The algorithmic approach uses an algorithm to construct a tree from the data. The tree-searching method constructs many trees, and then uses some criterion to decide which is the best or the best set of trees. Algorithmic methods are called also distance methods. Distances are expressed as the fraction of sites that differ between two sequences in a multiple alignment. If it is obvious that two sequences differing only 10% of their nucleotide sites are more closely than a pair differing 30%, it is not as obvious that the more time has passed since two sequences diverged the more they differ.

1.6.2.1 Distance methods

With distance method the evolutionary distances are computed for all pairs of taxa and the tree is constructed by considering the relationships among these determined distance values. The distances are expressed as the fraction of sites that differ between the two sequences.

Among distance methods, the unweighted pair-group (UPGMA) method measures the evolutionary distance, which is calculated for all pairs of taxa/sequences. This method is useful for constructing molecular phylogenies when rate of gene substitution is relatively constant (Nei & Kumar., 2000).

The Minimum Evolution (ME) method on the other hand, calculates the sum of all branch lengths estimates for all plausible trees and the tree that has the smallest sum is the tree that is chosen. Although accurate, this method is time consuming and for this reason has been almost completely replaced by the Neighbor-Joining (NJ) method (Nei & Kumar., 2000). The NJ method is based on the ME principle, but does not examine all the possible topologies. NJ method is currently the most popular algorithmic method. NJ manipulates a distance matrix, reducing it in size at each step, and then reconstructs the tree from that series of matrices. NJ is a minimum-changed method but it does not guarantee finding the tree with the smallest overall distance (Saitou & Nei., 1987).

1.6.2.2 Maximum parsimony

Maximum parsimony is based on the assumption that the most likely tree is the one that requires the fewest number of changes to explain the data in the alignment. The basic premise of parsimony is that taxa sharing a common characteristic do so because they inherited that characteristic from a common ancestor. When conflicts with that assumption occur, they are explained by reversal, convergence, or parallelism. These explanations are gathered together

under the term homoplasmy. Parsimony operates by selecting the tree or trees that minimise the number of evolutionary steps, including homoplasms, required to explain the data. Parsimony, or minimum change, is the criterion for choosing the best tree (Nei & Kumar., 2000). An algorithm is used to determine the minimum number of steps necessary for any given tree to be consistent with the data. That number is the score for the tree, and the tree or trees with the lowest scores are the most parsimonious trees (Nei & Kumar., 2000).

1.6.2.3 Maximum Likelihood methods

Maximum Likelihood (ML) tries to infer an evolutionary tree by finding that tree that maximises the probability of observing the data. For sequences, the data is the alignment of nucleotides or amino acids. Starting from one nucleotide position, the evolutionary model that gives the instantaneous rates at which each of the four nucleotides changes to each of the other three possible nucleotides, and a hypothetical tree of some topology and with branches of some length. Several scenarios exist, thus the probabilities of each of the scenarios must be determined and added together to obtain the probability of the tree (Nei & Kumar., 2000).

1.6.2.4 Estimating the reliability of a phylogenetic tree

A tree consists of two elements: nodes and branches. A branch is a line that connects two nodes. Nodes can be either external, which are the tips of the tree or internal, which are the points that represent a common ancestor of two other nodes.

Reliability in phylogenetics usually applies to the topology of the constructed tree. The most common way to determine reliability is bootstrapping. In bootstrapping, a random site is taken from an alignment and is used as the first site in pseudo-alignment. The process of comparison is repeated generally 100 to 1000 times, each time assigning a value (1 for complete correspondence, to 0 for not correspondence). In this way a tree with a 90% bootstrap score is more reliable than one with 70% (Nei & Kumar., 2000).

1.6.2.5 Bayesian analysis

Bayesian inference is based on the notion of posterior probabilities: probabilities that are estimated based on some model (prior expectations), after learning something about the data. Bayesian analysis of phylogeny is similar to ML in that the user postulates a model of evolution and the programme searches for the best trees that are consistent with both the model and the data (Nei & Kumar., 2000). ML seeks the tree that maximises the probability of observing the

data given that tree; Bayesian analysis seeks the tree that maximises the probability of the tree given the data and the model for evolution. However, it is usually not possible to calculate the posterior probabilities of all the trees analytically. The Markov chain Monte Carlo (MCMC) method, as implemented by the MrBayes program can be used for this purpose.

Like Parsimony and ML, Bayesian is applied to each site along the alignment. It begins with a tree with a combination of branch lengths, substitution parameters, and a rate variation across sites parameter to define the initial state of chain. A new state of a chain is then proposed and the probability of a new state, given the old state, is calculated. A random number between 0 and 1 is assigned, and if that number is less than the calculated probability, the new state is accepted. The program MrBayes deals with that by running several independent chains starting from the same initial tree. The chains quickly diverge from each other and one of them, the so-called “cold” chain, is the source of the tree that is saved typically once every 100 generations (Nei & Kumar., 2000).

1.6.3 Evolutionary models

Sequences diverge from a common ancestor because mutations occur and some fractions of those mutations are fixed into the evolving population by selection and by chance, resulting in the substitutions of one nucleotide for another at various sites. In order to reconstruct evolutionary trees, we must make some assumptions about that substitution process and state those assumptions in the form of a model (Nei & Kumar., 2000).

The easiest model to consider is one in which the probabilities of any nucleotide changing to any other nucleotide are equal. This simple model has only one parameter (the substitution rate), and is known as the one-parameter model or the Jukes-Cantor model (Nei & Kumar., 2000). Although the simplest, this method is non realistic. All changes may not occur at the same rates, and a variety of models have been proposed that allow us to specify different rates. The most general model is one in which each different substitution can occur at a different rate, which depends upon the equilibrium frequency that nucleotide. Other important models are all special cases of the above-mentioned general non-reversible method.

1.7 OBJECTIVES OF THE STUDY

The major objectives of the study were:

To explore the feasibility of applying novel molecular techniques to rabies diagnosis and typing.

To determine the molecular epidemiology and evolution of rabies viruses circulating in the reservoir species in Italy and Sub-Saharan Africa.

2 APPLICATION OF MOLECULAR METHODS TO IMPROVE RABIES DIAGNOSIS

2.1 DEVELOPMENT OF A ONE STEP RT-PCR FOR RABIES DIAGNOSIS IN ANIMALS AND HUMANS

De Benedictis P, De Battisti C, Dacheux L, Marciano S, Ormelli S, Salomoni A, Caenazzo ST, Lepelletier A, Bourhy H, Capua I, Cattoli G. Lyssavirus detection and typing using pyrosequencing. J Clin Microbiol. 2011 May;49(5):1932-8. Epub 2011 Mar 9.

2.1.1 Background

Reliable and rapid diagnosis is a prerequisite both to monitor lyssavirus distribution in animal reservoirs and to identify cases of rabies in humans. The use of a diagnostic method able to identify and distinguish among all lyssaviruses is particularly needed when imported cases occur in rabies-free areas (De Benedictis *et al.*, 2008), or when a lyssavirus succeeds in crossing the species barrier to infect unexpected novel host (Dacheux *et al.*, 2009). To date, the fluorescent antibody test (FAT) is the most widely adopted method for rabies diagnosis and is also the gold standard, followed by virus isolation on cell culture (rabies tissue culture isolation test or RTCIT) which should replace isolation in mice (mouse inoculation test or MIT), as recommended by international organizations (Anonymous , 2005, Anonymous , 2010). Molecular methods have been recently adopted as confirmatory assays or as first choice methods in those cases where *intra-vitam* diagnosis in humans is required. Molecular protocols dedicated to RNA viral detection demonstrate good performances for human rabies diagnosis and are currently adopted as diagnostic tools. However, all presently available methods have showed some limitations. In particular, hemi-nested (hn)RT-PCR methods are subjected to contamination events due to a further manipulation of samples. The efficacy of probe based real time RT-PCR is influenced by mutations arisen during viral replication or in case of occurrence of a new strain (Hoffmann *et al.*, 2010, Hughes *et al.*, 2004). Moreover, due to the variety of genus *Lyssavirus*, it is essential for a reliable molecular assay to be able to detect but also differentiate among existent rabies and rabies-related viruses.

In the present study a One-Step RT-PCR assay was developed to potentially and simultaneously detect and rapidly type all known lyssaviruses. The 3' terminus of the viral genome as target for the amplified product allowed to properly identify the virus involved in the infection, either by applying pyrosequencing or classical sequencing.

2.1.2 Materials and methods

2.1.2.1 Viruses and bacterial strains

Selected viruses and bacteria were used to test specificity and sensitivity of the One-Step RT-PCR assay. A panel of 18 lyssaviruses (including 7 species) were used in order to test the specificity of the method. In addition, 2 unassigned rhabdoviridae, 2 flaviviridae, 1 paramyxoviridae, 9 herpesviridae, 8 bacteria and 4 protozoans were also tested (Table 1). Viral working stocks for the standardization of the assay were produced on BHK-21 (ATCC n. CCL-10) and BSR cells (a clone of BHK-21 cells) respectively for RABV (CVS-11, batch Pd20) and EBLV-1 (8918FRA, batch Pd2). The median tissue culture infectious dose (TCID₅₀/ml) of virus used in the sensitivity tests was calculated according to the Spearman-Kärber formula (Lorenz & Bogel, 1973). Viruses other than lyssaviruses used in this study were propagated in different cell substrates: RK-13 (ATCC n. CCL-37) and equine derma primary cells for equine herpesviruses, Vero dogSLAM.tag cells (Seki *et al.*, 2003) for canine distemper virus, MDCK cells (ATCC n. CCL-34) for canine herpesvirus, and Vero cells (ATCC n. CCL-81) for flaviviruses. Human herpesviruses, bacterial and protozoan strains were provided by Dr. Jean-Claude Manuguerra (Institut Pasteur, Paris) and the Bacteriology and Parasitology Units (Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro, Italy), respectively.

Table 1: Viral and bacterial strains used in this study for specificity determination. Lyssaviruses belonging to different species and other pathogens commonly responsible for encephalitis in mammals were tested.

Family	Genus	Species	Strain (reference)	Host species/vector	Origin
Virus					
<i>Rhabdoviridae</i>	<i>Lyssavirus</i>	RABV	CVS-11	-	-
		RABV	GS7 1-11	Fox (<i>Vulpes vulpes</i>)	France
		RABV	Ariana 2	Dog (<i>Canis lupus familiaris</i>)	Tunisia
		RABV	Raccoon dog	Raccoon dog (<i>Nyctereutes procyonoides</i>)	Poland
		RABV	SAG2	Vaccine	France
		RABV	SAD-B19	Vaccine	Tubingen
		RABV	Bern-C	Vaccine	Opava
		RABV	SAD P5 (SAD 88)	Vaccine	Postdam
		RABV	ERA	Vaccine	-
		RABV	HEP	Vaccine	-
		RABV	LEP	Vaccine	-
		LBV	8619NGA	Bat (<i>Eidolon Helvum</i>)	Nigeria
		MOKV	86100CAM	Shrew (<i>Crocidura sp.</i>)	Cameroon
		DUVV	94286SA	Bat (<i>Minopterus sp.</i>)	South Africa
		EBLV-1 (subtype a)	122938	Bat (<i>Eptesicus serotinus</i>)	France
		EBLV-1 (subtype b)	121411	Bat (<i>Eptesicus serotinus</i>)	France
		EBLV-2	RV1332	Bat (<i>Myotis daubentonii</i>)	United Kingdom
		ABLV	9810AUS	Bat (<i>Pteropus sp.</i>)	Australia
	Unassigned (Le Dantec group)	LDV	Le Dantec virus DakHD 763 (9006SEN)	Human (<i>Homo sapiens</i>)	Senegal
	Unassigned (Ephemerovirus)	KOTV	Kotonkan virus Ib Ar23380 (9145NIG)	Dipteran	Nigeria
<i>Flaviviridae</i>	<i>Flavivirus</i>	TBEV	Tick-borne encephalitis virus, Hypr		
		WNV	West Nile virus, Eg 101		
<i>Paramyxoviridae</i>	<i>Morbillivirus</i>	CDV	Canine distemper virus, Arctic lineage		
<i>Herpesviridae</i>	<i>Varicellovirus</i>	CHV	Canine herpesvirus		
		EHV1	Equine herpesvirus 1		

		EHV4	Equine herpesvirus 4		
		SHV1	Aujeszky's disease virus		
		VZV	Varicella -zoster virus		
	<i>Simplexvirus</i>	HSV1	Herpes simplex virus type 1		
		HSV2	Herpes simplex virus type 2		
	<i>Cytomegalovirus</i>	HHV5	Human herpesvirus 5		
	<i>Rhadinovirus</i>	EHV5	Equine herpesvirus 5		
Bacteria					
		Leptospira spp.			
		Escherichia coli spp.			
		Brucella spp.			
		Campylobacter spp.			
		Salmonella spp.			
		Rickettsia spp.			
		Borrelia europea			
		Anaplasma spp.			
Protozoa					
		Atoxoplasma spp.			
		Toxoplasma spp.			
		Plasmodium spp.			
		Neospora spp.			

2.1.2.2 RNA extraction

Viral RNA was extracted from clinical samples and supernatants of cell cultures as previously described (Delmas *et al.*, 2008) or by using the Nucleospin RNA II kit, according to the manufacturer's instructions (Macherey-Nagel, Germany). For the latter, one hundred microlitres of sample suspension were used for the extraction, and RNA was eluted in a final volume of 60 μ l and stored at -80°C .

2.1.2.3 Primer set design

A lyssavirus specific primer set was designed on a relatively conserved region of the nucleoprotein (N) gene. This region was selected as prototype for lyssavirus typing and because of the relatively large availability of N gene sequences on GenBank database (accession numbers of complete sequences used for primer design are disclosed in Annex 1). A multiple alignment was performed using complete nucleotide sequences of the N gene available for historical and recent lyssaviruses, with a total of 76, 3, 4, 2, 6, 3 and 2 different sequences for RABV, LBV, MOKV, DUVV, EBLV-1, EBLV-2 and ABLV, respectively. Publicly available sequences of the recently identified bat lyssaviruses were also aligned. The expected 603 bp amplification products comprised the 3' terminus of the nucleoprotein gene, which has been widely used for lyssavirus classification as the most variable region among the N genes of known species (Kissi *et al.*, 1995). In order to be able to anneal with all the different species, the reverse primer encompassed 5 different polymorphisms, and was used as a mixture of three single primers, namely Rab Rev Pyro-biot1, 2 and 3, each harbouring 1, 2 and 2 distinct polymorphisms respectively (Table 2). Reverse primer mixture was then composed by an equal concentration of all the three primers, allowing for a better control the ratio of the different degenerated primers in the final mixture.

Table 2: Primer sequences targeting the lyssavirus nucleoprotein gene 3' terminus. Position referred to the challenge virus strain (CVS) of RABV (accession number GQ918139).

Primer	Sequence (5' - 3')	Position
RabForPyro	AACACYCTACAATGGA	59-75
RabRevPyro-biot 1	TCCAATTNGCACACATTTTGTG	662-641
RabRevPyro-biot 2	TCCARTTAGCGCACATYTTATG	662-641
RabRevPyro-biot 3	TCCAGTTGGCRCACATCTTGTG	662-641

2.1.2.4 One-Step RT-PCR

One-Step RT-PCR was performed using the OneStep RT-PCR kit, according to the manufacturer's instructions (Qiagen, Germany). The final primers concentration applied to PCR reaction was 400 nM. Five µl of isolated RNA were added to 45 µl of master mix, with a final volume of 50 µl. The following protocol was used: 30 min at 50 °C, 15 min at 95 °C followed by 45 cycles at 94 °C for 30 sec, 52 °C for 30 sec and 72 °C for 40 sec. Amplicons were detected by gel electrophoresis using 7% silver stained polyacrylamide gel.

2.1.2.5 Assay analytical specificity and sensitivity

The specificity of the primer set was tested on nucleic acids extracted from a panel of microorganisms, which may naturally cause encephalitis, or nervous signs of disease in mammals and humans (Table 1). Each strain was tested in triplicate.

The limit of detection (LoD) (EPA., 2004) of the One-Step RT-PCR protocol was evaluated by different approaches. First, RNA was extracted from cell culture supernatant containing ten-fold serial dilutions of RABV (CVS-11) and EBLV-1 (8918FRA) titrated according to the Spearman-Kärber formula (Lorenz & Bogel., 1973). Secondly, tests were performed on brain and saliva specimens to establish whether the sample matrixes could influence analytical sensitivity. Brains obtained from SPF mice were weighed (0.1gr) and homogenised with sterile quartz sand in 1 ml (1:10 w/v) of phosphate buffered saline (PBS, pH 7.4). The homogenates were then blended with the tested viruses serially diluted and processed for RNA extraction. Saliva was obtained from 6 healthy human volunteers, pooled and then blended with the tested viruses serially diluted and processed for RNA extraction. Each dilution was tested in

triplicates. The LoD of the method was defined as the last dilution at which at least 2 out of 3 replicates of each dilution were positive.

Finally, to determine the LoD of the RT-PCR protocol in terms of RNA copy numbers, *in vitro* transcribed RNA of 7 prototype species was analysed. Briefly, a segment of viral genome covering the entire ORF (Open Reading Frame) of the N genes of selected viruses was generated using primers specifically designed for each species and the amplification products (1480-1657 bp) were cloned into the pCR-II vector using the dual promoter TOPO TA Cloning® kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Recombinant plasmids were isolated from positive *E. coli* colonies using the GenElute™ Plasmid Miniprep kit (Sigma-Aldrich, St. Louis, USA). The synthetic N genes were sequenced using specific primers. The N insert control plasmids were linearized using the restriction enzyme *Hind*III (Roche Diagnostics, Penzberg, Germany) except for the plasmid with MOKV insert, which was linearised with *Bam*HI (New England Biolabs, MA, USA). The *in vitro*-transcribed RNA was obtained from the T7 promoter using the MEGAscript T7 kit (Ambion, Inc) according to the manufacturer's recommendations and then quantified by UV BioPhotometer (Eppendorf, Hamburg, Germany). The number of the RNA copies was calculated according to the formula reported in a previous study (Fronhoffs *et al.*, 2002). Ten-fold dilutions of the RNA transcripts, ranging from 10^8 to 10^{-1} copies/μl, were prepared. Independent triplicates determined the LoD of the assay.

2.1.2.6 Clinical sensitivity and specificity of the assay

To evaluate whether or not the RT-PCR assay could be used as a reliable tool for rabies diagnosis, 897 samples collected from animals (n=882) and humans (n=12) between 1992 and 2010, and from cell culture supernatants (n=3) were retrospectively analysed. Samples consisted of brain tissues from clinical samples (n=782) and from mice inoculated intra cerebrally (n=42) with fixed strains or field samples, salivary glands (n=1), oral swabs (n=24) and blood clots (n=10) from bat surveillance, human saliva (n=9), skin biopsies from naturally (n=3) and experimentally infected animals (n=23), and cell culture supernatants (n=3). Brain samples from 28 mammalian species (including humans) were tested (see Table 3). The strains responsible for the infection belonged to 7 lyssavirus species, representative of all the 5 continents (see Annex 2).

Assay performances were compared to FAT, as gold standard test. Clinical sensitivity (Se) and specificity (Sp) and their confidence interval (CI - 95%), were calculated between the One-Step

RT-PCR developed and FAT as the gold standard test. The McNemar test and Cohen's agreement value (K) were then calculated (Everitt, 1989).

2.1.2.7 Comparison with hnRT-PCR

A total of 45 brain samples and 23 skin biopsies were analysed in parallel by the One Step RT-PCR assay developed and the well-established hnRT-PCR previously described (Dacheux *et al.*, 2008). The protocol described by Dacheux and colleagues (Dacheux *et al.*, 2008) was developed and standardized in three laboratories, one in France and two in developing countries where rabies is still endemic (Cambodia and Madagascar) and it is currently used to diagnose human rabies (*intra-vitam* and *post-mortem*) using saliva, urine, and skin biopsy specimens.

2.1.3 Results

2.1.3.1 Analytical laboratory evaluation

The primer set designed was firstly tested *in silico*, using Mega4 program (Tamura *et al.*, 2007) and was able to anneal to all sequences analysed, including those from putative new species. Besides, primer set designed was also able to detect RNA of all 7 lyssavirus species tested. No positive results were obtained with any of the other organisms listed in Table 1, indicating high specificity.

In terms of N gene copy number, the LoD was 10 gene copies/ μ l of starting material of *in vitro*-transcribed RNA for RABV, MOKV, EBLV-1 and EBLV-2, 10^2 gene copies/ μ l for DUVV and ABLV, and 10^3 gene copies/ μ l for LBV. The LoD of the method relative to infectious virus titre detectable ranged between $0.5 \cdot 10^{-0.2}$ and $0.5 \cdot 10^{1.24}$ TCID₅₀/ml for CVS-11 and EBLV-1, respectively (Table 4). The alignment between the sequences of the mixture of degenerated reverse primers and of the synthetic N genes showed a total of mismatches less or equal to 6 for species RABV, MOKV, EBLV-1 and EBLV-2, which were detected at a concentration of 10 gene copies/ μ l. Total mismatches observed were 7 for LBV, DUVV and ABLV, for which LoD ranged between 10^2 to 10^3 gene copies/ μ l.

Table 3. Summary of samples analysed in this study.

Species	Saliva/ Oral Swabs	Salivary glands	Brain	Blood Clots	Skin biopsy	Cells/ supernatant	Samples per species
Fox (<i>Vulpes vulpes</i>)			521				521
Cat (<i>Felis catus</i>)			89				89
Dog (<i>Canis lupus familiaris</i>)			67				67
Mouse (<i>Mus musculus</i>)			42		23		65
Bat (<i>Myotis myotis</i>)	10			10			20
Bat (<i>Myotis capaccinii</i>)	9						9
Bat (<i>Eptesicus serotinus</i>)	5						5
Badger (<i>Meles meles</i>)		1	20		3		24
Roe deer (<i>Capreolus capreolus</i>)			18				18
Raccoon dog (<i>Nyctereutes procyonoides</i>)			16				16
Stone marten (<i>Martes foina</i>)			10				10
Bovine (<i>Bos taurus</i>)			9				9
Human (<i>Homo sapiens</i>)	9		3				12
Caprine (<i>Capra hircus</i>)			6				6
Deer (<i>Cervus elaphus</i>)			5				5
Donkey (<i>Equus asinus</i>)			2				2
Ovine (<i>Ovis aries</i>)			2				2
Horse (<i>Equus caballus</i>)			1				1
Mouflon (<i>Ovis musimon</i>)			1				1
Ibex (<i>Capra ibex</i>)			1				1
Squirrel (<i>Sciurus vulgaris</i>)			1				1
Hare (<i>Lepus europaeus</i>)			1				1
Rat (<i>Rattus norvegicus</i>)			1				1
Polecat (<i>Mustela putorius</i>)			1				1
Rabbit (<i>Oryctolagus cuniculus</i>)			1				1
Chamois (<i>Rupicapra rupicapra</i>)			1				1
Honey badger (<i>Mellivora capensis</i>)			1				1
Jackal (<i>Canis aureus</i>)			1				1
Lion (<i>Panthera leo</i>)			1				1
Wildcat (<i>Felis silvestris silvestris</i>)			1				1
Prairie dog (<i>Cynomys gunnisoni</i>)			1				1
Cells/cell supernatants						3	3
Total	33	1	824	10	26	3	897

Table 4. Limit of detection of the One-Step RT-PCR tested for RABV and EBLV-1 using cell culture supernatants, brain homogenates and saliva as biological matrices.

Species	Strain	Titre of virus stock (TCID ₅₀ /ml)	Biological matrix	Sensitivity (TCID ₅₀ /ml)
RABV	CVS-11	10 ^{5.8}	cell culture supernatant	0.5*10 ^{-0.2}
			brain	0.5*10 ^{-0.2}
			saliva	0.5*10 ^{-0.2}
EBLV-1	8918FRA	10 ^{6.24}	cell culture supernatant	0.5*10 ^{1.24}
			brain	0.5*10 ^{1.24}
			saliva	0.5*10 ^{1.24}

2.1.3.2 Clinical detection of viral RNA in field and experimental samples

A total of 824 samples was analysed by FAT, as gold standard for rabies diagnosis and by the One-Step RT-PCR object of this study (Table 5). One out of the 824 analysed samples resulted negative by this method and positive by FAT. Eight samples resulted positive by this method but were not confirmed by the standard method FAT. All of them were further confirmed as RABV by both pyrosequencing and classical sequencing. The hnRT-PCR (Dacheux *et al.*, 2008) was applied to those 8 samples, confirming 4 of them as positive.

The One-Step RT-PCR showed high relative specificity (98.94%, C.I. 97.55-99.65) and sensitivity (99.71%, C.I. 98.40-99.99) values in comparison with those obtained with the FAT used as gold standard method. Accuracy of method was calculated as 98.90%. The agreement between the One-Step RT-PCR developed and the gold standard method (FAT) was calculated as 98.91% with Cohen's kappa coefficient 0.977, which corresponds to an almost perfect agreement between the two methods. The difference between them calculated by applying the McNemar test was considered statistically significant ($\chi^2 = 5.44$), as it indicated that the different results obtained were due to intrinsic characteristics of the two methods.

A total of 68 samples were tested in parallel by the OneStep RT-PCR assay (including the 9 previous specimens for which discrepant results were obtained with FAT) and the previously described hnRT-PCR (Dacheux *et al.*, 2008). 62 of them (91.18%) showed complete agreement between the two methods (Table 5).

Table 5. Summary of samples analysed by the ONE-Step RT-PCR and FAT, as gold standard (a), or the ONE-Step RT-PCR and the previously published hnRT-PCR method (b) (Dacheux *et al.*, 2008).

a)

ONE-Step RT-PCR	FAT		Total
	(-)	(+)	
(-)	468	1 ^c	469
(+)	8 ^d	347	355
Total	476	348	824

b)

ONE-Step RT-PCR	hnRT-PCR		Total
	(-)	(+)	
(-)	0	1 ^c	1
(+)	5 ^d	62	67
Total	5	63	68

c: identical sample.

d: 4 out of them were identical samples.

2.1.4 Discussion

To date, real-time and (hemi) nested RT-PCR have been favoured to diagnose rabies as they provide a better performance if compared to end-point not nested protocols. However, the One-Step RT-PCR developed in this study demonstrated high sensitivity and specificity levels and a high value of accuracy, as well as in previously published methods. The method was in fact capable to detect less than 1 TCID₅₀/ml and 10 gene copies/μl of synthetic RNA of RABV.

Only 9 discrepant results out of the analysed 897 samples using multiple diagnostic tests were detected (see Table 5). This occurrence is a rather expected event when a large set of clinical samples are included in a validation process and this could have several explanations, such as the different targets and sensitivity levels of the applied protocols. Molecular methods are able to reveal low infection levels, as well as low levels of contamination potentially occurring during the diagnostic process, from the collection of samples to the final step of amplification.

Although the occurrence of detecting failures could not be completely excluded, the use of the OneStep RT-PCR presents the advantage of being a mutation resistant method, since it is based on primers designed on conserved regions. This is in contrast with probe-based methods, such as real-time PCR, where results can be negatively affected by the occurrence of viral mutations since the design of the probe is on the most variable region.

Moreover, the method developed has the advantage of being applicable to a variety of samples of human and animal origins and represents the main diagnostic tool when standard protocols are not applicable, i.e. *intra vitam* diagnosis. The majority of samples analysed were brain specimens, and only 70/897 samples were from different matrices (see Table 3). However, the analytical sensitivity of the method has been tested on saliva, showing the same sensitivity values as for brain specimens and cell culture supernatants (see Table 4). In addition, a panel of skin biopsies were also analysed in parallel comparing this method to the previously described hnRT-PCR (Dacheux *et al.*, 2008) obtaining an almost perfect agreement (see Table 5 and Annex 2). Only 33 saliva/oral swabs and 26 skin biopsy samples were tested in this study (see Table 3). However, it should also be taken into account that these matrices are not regularly submitted to the laboratories and therefore they are not easily available for validation purposes.

Over 300 RABV representatives from 4 different continents, and strains belonging to LBV, MOKV, DUVV, EBLV-1, EBLV-2 and ABLV were analysed. Moreover, results from *in silico* analyses clearly indicate that the method has the potential to detect all known lyssaviruses including those belonging to putative new species. A further screening of a wider panel

representative of the worldwide diversity of known and emerging lyssaviruses should be applied in order to assess the performances of the method developed in this study. Improvements and subsequent re-validation of molecular diagnostic protocols should always be taken into consideration, not only for rabies but also in case of other viral diseases (Cattoli *et al.*, 2009, Coertse *et al.*, 2010, Hoffmann *et al.*, 2010, Nadin-Davis *et al.*, 2009, Xing *et al.*, 2008). The One-Step RT-PCR developed was validated in compliance with international guidelines for diagnostic molecular techniques (Anonymous , 2005, Anonymous , 2010). The method can be notably used in combination to standard methods as an early warning detection tool, since it is capable of rapidly revealing the emergence or the introduction of a novel lyssavirus species in a given susceptible population or geographical area.

2.2 APPLICATION OF PYROSEQUENCING TO RABIES TYPING

2.2.1 Viral typing for all known lyssaviruses

De Benedictis P, De Battisti C, Dacheux L, Marciano S, Ormelli S, Salomoni A, Caenazzo ST, Lepelletier A, Bourhy H, Capua I, Cattoli G. Lyssavirus detection and typing using pyrosequencing. J Clin Microbiol. 2011 May;49(5):1932-8. Epub 2011 Mar 9.

2.2.1.1 Background

Several PCR methods have been developed over the past decades for the detection or confirmation of a rabies infection. Although gel-based systems have the advantage of being not affected to genome mutations, the detection of amplified RT-PCR products, without further sequencing, should be interpreted with caution, with high risk of generating cross-contamination, especially when using hemi-nested RT-PCRs, (Belak & Thoren., 2001) (Robardet *et al.*, 2011). The importance of sequencing the PCR products was highlighted in an experimental study, which notified the finding of host genomic amplicons of the same size as the target amplicons were observed on the agarose gels, which were subsequently confirmed as false positives following direct sequencing (Hughes *et al.*, 2006). Many laboratories now use partial sequencing to confirm the detection of a lyssavirus and obtain data that can be used in a phylogenetic analysis of viruses circulating in a specific region. However, the requirement for further genomic sequencing increases 'time' and 'final' cost of the complete analysis in order to clearly define the species involved in the infection.

Pyrosequencing has proved to be a reliable alternative sequencing approach applicable to whole genome sequencing as well as to pathogens typing (Nyren., 2007). Its use is currently applied to a variety of diagnostic procedures, such as the human papillomavirus and the hepatitis C typing (Ronaghi & Elahi., 2002, Swan *et al.*, 2006). The aim of this work was therefore to apply pyrosequencing to the amplified products obtained by the One Step RT-PCR described in chapter 2.1 and to compare the results to those of classical sequencing in terms of accuracy as well as time and costs.

2.2.1.2 Materials and methods

2.2.1.2.1 Viruses and bacterial strains.

Selected viruses and bacteria used were listed in the chapter 2.1.2.1. See table 1 and Annex 2 to further details.

2.2.1.2.2 Primer set design

The primer set designed has been described in chapter 2.1.2.2. Particularly, the forward primer used for both PCR amplification and pyrosequencing reactions was designed on top of the starting codon of the N gene so that the further 30 bp sequence obtained through pyrosequencing allows a rapid typing of the viral strain analysed among the existent lyssavirus species.

2.2.1.2.3 Sequencing and pyrosequencing

Pyrosequencing protocol was performed following the previously described steps (Marsh., 2007, Pizzuto *et al.*, 2010). Briefly, biotinylated amplicons were detected by gel electrophoresis using 7% silver stained polyacrylamide gel, further captured with streptavidin sepharose beads and purified with a vacuum prep workstation according to the manufacturer's instructions (Biotage, Uppsala, Sweden). Single-stranded biotinylated DNAs were then transferred to 40 µl annealing buffer containing pyrosequencing primers at a final concentration of 0.5 µM. Pyrosequencing reactions were performed using the Pyromark ID platform (Biotage, Uppsala, Sweden). The quality of pyrograms of 30-40 bp was satisfactory in all the tested samples.

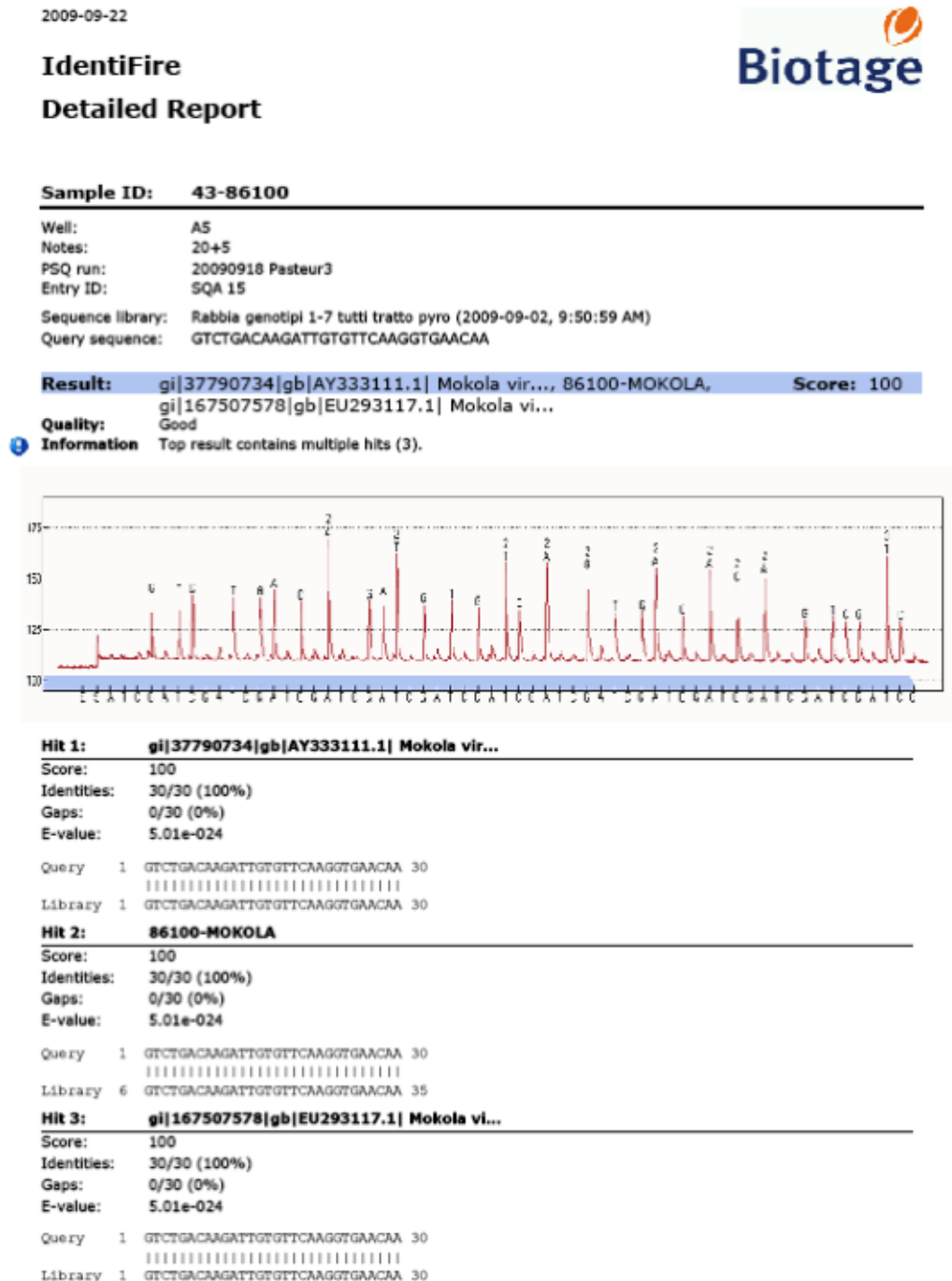
The conventional sequencing method was also applied by using the amplified product obtained by the One-Step RT-PCR assay. Briefly, 603 bp sequences were generated using the Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystem, Foster City, CA - USA) and primers used in the One-Step RT-PCR assay. The products of the sequencing reactions were cleaned-up using PERFORMA DTR Ultra 96-Well kit (Edge BioSystems, Gaithersburg, MD - USA) and sequenced in a 16-capillary ABI PRISM 3130xl Genetic Analyzer (Applied Biosystem, Foster City, CA - USA).

2.2.1.3 Results

Pyrosequencing was applied to all 375 samples tested positive by the One Step RT-PCR. Using the pyrosequencing approach, taxonomic classification of all seven lyssavirus species was possible (Annex 2). Results obtained by using pyrosequencing were directly analyzed by the software and clearly reported as an Identifire Summary Report (Identifire Software, Biotage,

Uppsala Sweden) (See Figure 5). All pyrosequencing results showed complete agreement with Sanger method sequencing.

Figure 5. IdentiFire result. The sequence obtained is automatically blasted to a library of sequences previously selected. The library is upgradable with newly produced / published sequences.



2.2.1.4 Discussion

In the present study a pyrosequencing protocol was developed to simultaneously and rapidly

type all known lyssaviruses. The 3' terminus of the viral genome as target for the amplified product allowed to properly identify the virus involved in the infection, either by applying pyrosequencing or classical sequencing. The pyrosequencing protocol could easily differentiate among lyssavirus species and results were in complete agreement with the Sanger sequencing method.

Several advantages characterize this newly developed method, such as the rapidity in obtaining a final diagnosis, the lower cost and the possibility to directly identify the lyssavirus species involved in the infection.

A maximum time span of about 5 hours from the submission to the final typing of a positive field sample is needed to analyze a suspect sample in laboratory conditions. Starting from the amplified products, a pyrosequencing analysis requires only 2 hours to obtain the final result; almost half the time compared to classical Sanger sequencing.

Pyrosequencing analysis does not need any terminator enzymes or prior purification steps, thus resulting in limited costs per analysis if compared to classical sequencing. In fact, the average cost (calculation made according to European standards) for the developed pyrosequencing protocol has been estimated between 3.87 to 6.03 euro/sample (for 96 or 25 samples analyzed/run, respectively), compared to 9.86 euro/reaction for the classical sequencing method.

2.2.2 Viral typing applied to DIVA strategy (differentiating infected from vaccinated animals)

De Benedictis P, De Battisti C, Marciano, Mutinelli F, Capua I, Cattoli G. A novel high-throughput molecular method reveals no evidence of vaccine-associated rabies cases following oral vaccination in Italy, 2008-2011. Submitted.

2.2.2.1 Background

Terrestrial rabies in carnivores is caused by the *Rabies virus* (RABV) species and is the main cause of human cases, leading to about 55,000 deaths per year (Knobel *et al.*, 2005). Rabies can be controlled or eradicated by implementing vaccination measures. To date, oral rabies vaccination (ORV) campaigns have been applied to various wildlife species in the Americas (reviewed in (Slate *et al.*, 2009)), and have successfully eliminated rabies among red foxes (*Vulpes vulpes*) in several European countries. Control programs are continuing to expand into Eastern Europe (Blancou., 2008) .

Although ORV represents an effective tool for rabies control, the rare occurrence of vaccine-associated rabies cases in animals has been described for replication-competent vaccine strains, including the Evelyn Rokitnicki Abelseth (ERA) BHK21 in Canada and Street Alabama Dufferin (SAD) Bern in Switzerland, and more recently for the SADB19 and SADP5/88 in Germany and Austria (Fehlner-Gardiner *et al.*, 2008, Muller *et al.*, 2009, Wandeler., 1988). Experimental studies have shown that SADB19 is safe in six species of carnivores but can maintain residual pathogenicity in rodents and at high doses in skunks (Vos *et al.*, 1999, Vos *et al.*, 2002). SADB19 has also proven to be genetically stable after serial intra cranial passages in mice and foxes (Beckert *et al.*, 2009).

Mutated vaccines such as Street Alabama Gif (SAG) 1 and 2 have been developed with an amino acid mutation at the arginine 333 codon to glutamic acid (R333E), previously identified as a determinant of attenuation (Dietzschold *et al.*, 1983, Lafay *et al.*, 1994). SAG2 has been proven safe for several species (Bingham *et al.*, 1997, Hammami *et al.*, 1999), although its stability following serial passages has never been experimentally demonstrated nor monitored in the field.

Given the well known residual virulence for ERA and SAD-Bern derivatives, international organizations have strongly recommended monitoring the occurrence of vaccine-associated rabies in areas subject to ORV campaigns (EU European Commission., 2002, WHO World Health

Organization., 1989). In North-Eastern Italy, following the re-introduction of fox rabies in October 2008 (De Benedictis *et al.*, 2008), several oral vaccination campaigns were implemented to control the disease in the animal reservoir. During these campaigns two different rabies vaccine viruses were used, namely SAG2 (SAG2-Rabigen, Virbac) and SADB19 (Fuchsoral, IDT Biologika). A total of 1,792,000 SAG2 and 280,500 SADB19 baits were distributed aurally and, to a lesser extent, manually, covering a maximum area of approximately 300,000 km², as of August 2011 (Figure 6 and Annex 3).

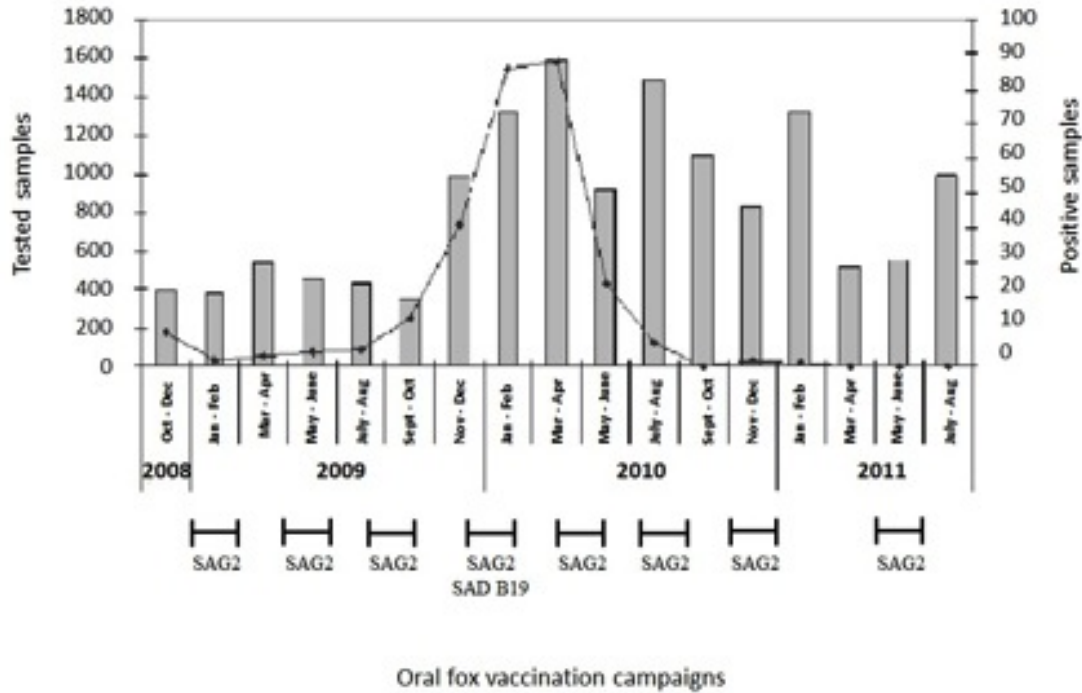
In order to investigate the occurrence of vaccine-associated cases, a high-throughput method for rapid screening of field samples collected during ORV surveys was developed and applied to all rabies specimens collected in Italy during the 2008-2011 epidemic prior to and following the implementation of ORV campaigns that used either SAG2 and SADB19 vaccine baits.

2.2.2.2 Materials and methods

2.2.2.2.1 Fixed viruses and field samples

A panel of 305 RABV obtained from clinical cases, plus six replication-competent vaccine strains including ERA, SAD Vnukovo, SAD Tubingen, SAD Postdam, SADB19 and the mutated strain SAG2, were included in the study. The SADB19 and SAG2 strains contained in vaccine baits distributed in Italy were tested without any further passage while the remaining live attenuated viruses were previously passaged in mice (Annex 4). All rabies-positive Italian field samples (n= 287) collected between early 2008 and February 2011 were tested, including those from wildlife and domestic animals, from a total of 12 animal species (Annexes 3, 4 and Figure 6). The remaining 18 field samples represented viruses collected from 12 countries spanning four continents.

Figure 6. Temporal distribution of the samples analysed for rabies and the ORV campaigns from October 1st 2008 to September 1st, 2011. Black line: positive cases detected and analysed in the study for the occurrence of vaccine-associated rabies. Vaccination campaigns and vaccine used are indicated at the bottom.



2.2.2.2.2 Primer set design

Based on the literature and sequences available in public data bases (Geue *et al.*, 2008), a lyssavirus specific primer set was designed for the gene encoding for the glycoprotein (G). A multiple alignment of 198 complete G gene nucleotide sequences available from historical and recent RABVs and from replication-competent vaccine strains was performed. The expected 230 bp amplification product spanned residues 4293 to 4522 of the G gene, corresponding to antigenic site III of the glycoprotein (see Table 6 and Annex 5).

A sequencing primer was then designed so that the 30 bp sequence obtained from pyrosequencing would include different sites of significance for strain discrimination: amino acid residue 333 (CGG or AGA to GAA in SAG2, corresponding to nucleotide residues 4373-4375) and two vaccine strain-associated polymorphisms at residues 4391 and 4396 selected according to the sequence alignment analysis (accession number GQ918139, A4391C and C4396T) (see Table 6 and Annex 5).

Table 6: Primer sequences targeting the RABV nucleoprotein gene 3' terminus. Position referred to the challenge virus strain (CVS) of RABV (accession number GQ918139).

Primer	Sequence (5'- 3')	Position
RV_G_Pyro For	AACTTGTCCTCCYGGGTTYGG	4293-4311
RV_G_Pyro Rev	CTGRAGGAGGGATGAYTGC	4522-4504
RV_G_Pyro Seq	YGATGCYCACTACAAGTCAG	4351-4370

2.2.2.2.3 One-Step RT-PCR and sequencing

Viral RNA was extracted from clinical samples and supernatants of cell cultures as previously described (Delmas *et al.*, 2008), or using the NucleoSpin® RNA II kit according to manufacturer's instructions (Macherey-Nagel, Germany). One-Step RT-PCR was performed using the Qiagen OneStep RT-PCR kit, according to manufacturer's instructions (Qiagen, Germany). The final concentrations applied were 200 nM for the two primers and 400 µM for the dNTPs. Five microlitres of isolated RNA were added to 45 µl of master mix, with a final volume of 50 µl. The following optimized protocol was used: 30 min at 55 °C, 2 min at 94 °C followed by 15 cycles at 94 °C for 30 sec, 60 °C for 30 sec and 68 °C for 30 sec, 15 cycles at 94 °C for 30 sec, 58 °C for 30 sec and 68 °C for 30 sec, and 15 cycles at 94 °C for 30 sec, 57 °C for 30 sec and 68 °C for 30 sec. Final extensions were done for 5 min at 68°C.

The pyrosequencing protocol was performed as described in paragraph 2.2.1.2.3 using the Pyromark ID platform (Biotage, Uppsala, Sweden).

The Sanger sequencing method was also applied to the amplified product (230bp), using the Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystem, Foster City, CA-USA) in a 16-capillary ABI PRISM 3130xl Genetic Analyzer (Applied Biosystem, Foster City, CA-USA) to confirm the pyrosequencing results.

2.2.2.3 Results

In silico, the primers were virtually able to hybridize to all sequences available on the public database (n=198, GenBank) and aligned using Mega4 program (Tamura *et al.*, 2007). All 305 field samples and 6 vaccine strains included in this study were subsequently tested in the laboratory using these primers. The quality of pyrograms of 30-40 bp was satisfactory in all

samples tested (i.e. score $\geq 90\%$) and the results obtained were directly analysed by the software and reported as an Identifire Summary Report (Identifire Software, Biotage, Uppsala Sweden). A clear differentiation of wild from vaccine strains was obtained according to the presence of the amino acid mutation R333E for SAG2 strains and of two nucleotide polymorphisms A4391C and C4396T in all vaccine strains, including SAG2, SAD and ERA. All 287 Italian cases were confirmed as street viruses, based on the absence of the selected polymorphisms in the sequences obtained (Annexes 4 and 5). All pyrosequencing results were in complete agreement with Sanger method.

2.2.2.4 Discussion

The risk of vaccine-associated rabies cases has long been recognised and the World Health Organization has recommended monitoring of its occurrence through the application of monoclonal antibody typing (WHO World Health Organization., 1989). However, it has been recently pointed out that this protocol can fail, thus leading to an underestimation of the real burden of vaccine-associated cases (Muller *et al.*, 2009). As for other infectious diseases, genetic sequencing and molecular techniques are increasingly being applied for the confirmation and typing after a first rabies diagnosis in a given area (Fooks *et al.*, 2009). In recent years, genetic characteristics of vaccine strains have been studied and made publicly available (Geue *et al.*, 2008). In this study a pyrosequencing protocol was developed as a useful diagnostic tool for ORV campaign surveys to rapidly distinguish between vaccine and street RABVs. The pyrosequencing method has several advantages, such as its rapidity and low cost (see paragraphs 1.7.1.2 and 2.2.1.4), and could be adopted as a high-throughput alternative to conventional methods. The protocol described herein has been developed as a “proof of principle” of the feasibility of applying pyrosequencing on such a large-scale survey. This method represents a rapid screening for field samples tested positive by standard methods and it has been directly applied in an ORV survey for vaccine-associated cases.

By applying this protocol all the sequences obtained from a panel of selected samples collected worldwide have been correctly identified. The primer set has been further tested *in silico* on a large number of sequences. By targeting three polymorphisms, namely the amino acid mutation R333E previously documented for SAG2 and the A4391C and C4396T nucleotide substitutions observed uniquely in the vaccine sequences of SAG2, SAD and ERA strains, we were able to discriminate between vaccine and street viruses. The fact that non-sense polymorphisms at amino acid position 333 were found in both field and vaccine strains except

for SAG2, indicates this codon should not be used as the unique discriminant site for diagnostic purposes. On the contrary, the other two non-sense polymorphisms (A4391C and C4396T) were present only in the vaccine strains analysed but absent in all field strain sequences available in public databases (n=198). Therefore, the polymorphisms observed can be used in combination to guarantee a correct classification of a virus responsible for the infection. Due to the variability of the G genes, sequencing a larger panel of strains is recommended to confirm this data.

In Italy, more than 14,000 samples have been screened for rabies over the last three years since the beginning of the 2008 epidemic, using FAT alone or in combination with RTCIT and RT-PCR (Anonymous, 2010, De Benedictis *et al.*, 2011), with 287 cases identified (as of August 2011) (see figure 4 and Annexes 3 and 4). All rabies viruses detected in Italy have been typed as wild type strains, with no vaccine strains having reverted to virulent forms. This is consistent with previous field and experimental reports on the rare occurrence of SAD-derived rabies cases and on the absence of vaccine-associated cases following the use of SAG2 (Bingham *et al.*, 1997, Muller *et al.*, 2009).

However, considering the rare occurrence of vaccine-associated cases in the field (Fehlner-Gardiner *et al.*, 2008, Muller *et al.*, 2009), a very high number of samples should be processed and post-vaccination surveys must be prolonged over the whole duration of ORV campaigns in order to confirm these findings. This is also the rationale behind the development of accurate and cost-effective protocols adaptable to high-throughput.

To date, few documented reports exist on the assessment of vaccine-associated cases following ORV. In Canada, 134,000 animals were tested over a period of 16 years and more than 9,500 rabies cases were registered, including nine vaccine-associated cases in 4 species of wild and domestic animals (Fehlner-Gardiner *et al.*, 2008). In Germany, 401 rabies cases were detected out of more than 51,000 tested animals over a ten year-surveillance, with a total of 4 being vaccine-associated (Muller *et al.*, 2009). Muller and colleagues (Muller *et al.*, 2009) have recently reported that fluorescence antibody tests (FAT) failed in detecting 3/6 vaccine-associated cases in Germany and Austria, likely due to the low level of infection in the Central nervous system.

The present investigation describes the development of a high-throughput method for vaccine virus typing as an alternative to monoclonal antibody typing or Sanger sequencing, which could be used to complement other pyrosequencing based protocols for rapid rabies detection and typing (De Benedictis *et al.*, 2011). Similar pyrosequencing approaches can be developed

for post-vaccination surveys of other infectious diseases in both wildlife and domestic animals, for which attenuated vaccines are used (i.e. classical swine fever in wild boars and pseudorabies in pigs (Pensaert *et al.*, 2004, Uttenthal *et al.*, 2010)).

3 MOLECULAR EPIDEMIOLOGY AND EVOLUTIONARY ANALYSES OF RABIES VIRUSES CIRCULATING IN DOMESTICATED AND WILDLIFE ANIMALS

3.1 MOLECULAR EPIDEMIOLOGY AND EVOLUTIONARY ANALYSES OF DOG RABIES CIRCULATING IN WEST AND CENTRAL AFRICA

Talbi C, Holmes EC, De Benedictis P, Faye O, Nakouné E, Gamatié D, Diarra A, Elmamy BO, Sow A, Adjougou EV, Sangaré O, Dundon WG, Capua I, Sall AA, Bourhy H. Evolutionary history and dynamics of dog rabies virus in Western and Central Africa. J Gen Virol. 2009 Apr;90(Pt 4):783-91.

3.1.1 Background

The burden of rabies in Africa is second globally behind Asia, with around 24,000 human deaths estimated each year (Knobel *et al.*, 2005). Dogs (*Canis familiaris* L.) have always been the principal host species of rabies throughout this area (Nel & Markotter., 2007).

Among the biodiversity of Lyssaviruses, three species (RABV, LBV and MOKV) circulate in Africa with dog-adapted viruses belong to RABV. Previous molecular epidemiological studies of rabies in Africa (Bourhy *et al.*, 2008, David *et al.*, 2007, Kissi *et al.*, 1995) have revealed that at least four clades of RABV circulate in this continent.

The Africa 1 clade, adapted to dogs, was the most similar to current Eurasian RABV lineages and therefore was grouped into a larger 'Cosmopolitan' clade (Kissi *et al.*, 1995) (Swanepoel *et al.*, 1993), together with a novel clade (Africa 4) recently identified in Egypt (Bourhy *et al.*, 2008, David *et al.*, 2007). In contrast, the African 3 clade is restricted to Southern Africa and is adapted to mongoose, so that it constitutes an epidemiological cycle distinct from that of dog rabies (Bourhy *et al.*, 2008, David *et al.*, 2007, Kissi *et al.*, 1995, Van Zyl *et al.*, 2010). Finally, the Africa 2 clade includes strains that circulate in dogs in several Central and Western African countries.

The dynamics of RABV in domestic dog populations, such as the diversity, distribution and origin of dog RABV circulating in Western and Central Africa have been poorly investigated. Prior studies were either geographically restricted or limited to a very small region of the viral genome, and compromised by a small sample size and a lack of exact spatial co-ordinates (Durr *et al.*, 2008, Kissi *et al.*, 1995, Smith *et al.*, 1993).

In the present study, phylogenetic and coalescent approaches have been combined to infer key aspects of viral phylogeography, especially spatio-temporal dynamics, of dog RABV over a large geographic area covering West and Central Africa.

3.1.2 Material and methods

3.1.2.1 Viruses and sequencing

A total of 92 sequences, 1335 nt in length, of the N gene were obtained from specimens collected from 27 countries over a time period of 29 years. For these sequences, the time (year) of sampling was available, as well as a spatial co-ordinate for the majority of them. These sequences covered. Relevant epidemiological information for all those RABV isolates analysed in this study is presented in Annex 6.

Viral RNA was extracted from clinical samples and supernatants of cell cultures as previously described (Delmas *et al.*, 2008) or by using the Nucleospin RNA II kit, according to the manufacturer's instructions (Macherey-Nagel, Germany). For the latter, one hundred microlitres of sample suspension were used for the extraction, and RNA was eluted in a final volume of 60 µl and stored at -80 °C.

RT-PCR and sequencing reactions were performed as described previously (Bourhy *et al.*, 2008). Additional primers used in this study were N127 (5'-ATGTAACACCTCTACAATGG-3'), N8m (5'-CAGTCTCYTCNGCCATCTC-3'), N42 (5'-CACATYTTGRTGAGTTGTCA-3') and N577 (5'-AAGATGTGYGCYAAAYTGGAG-3'), positions 55-74, 1568-1576, 633-651, 644-663, respectively, of the lyssavirus genome (Delmas *et al.*, 2008).

3.1.2.2 Evolutionary analysis

The N gene sequences newly described here were combined with relevant sequences from GenBank, resulting in two data sets of (i) 97 sequences from the Africa 1 and Africa 2 clades combined, and (ii) 134 Africa 2 sequences. For each data set, a Maximum Clade Credibility (MCC) phylogenetic tree was inferred, using the Bayesian Markov Chain Monte Carlo (MCMC) method available in the BEAST package (Drummond & Rambaut, 2007), thereby incorporating information on sampling time. Posterior probability values provide an assessment of the degree of support for each node on the tree. This analysis utilized a relaxed (uncorrelated lognormal) molecular clock and the HKY85+Γ₄ model of nucleotide substitution, although highly similar results were obtained under more complex substitution models. As demographic history can be considered a nuisance parameter in our study, we utilized the Bayesian skyline model as a coalescent prior. All chains were run for a sufficient length to ensure convergence with 10% removed as burn-in. As well as generating the MCMC tree, this same analysis also allowed us to estimate both the rate of nucleotide substitution per site

(subs/site/year) and the Time to Most Recent Common Ancestor (TMRCA) in years. The degree of uncertainty in each parameter estimate is provided by the 95% HPD (highest posterior density) values.

A parsimony-based approach was applied to determine the geographic structure of dog RABV of Africa 2, based on the MCC tree determined above, and with the country from where each sequence was collected as the unit of analysis (although this broad-scale categorization clearly hides a great deal of geographical structure). The number of unambiguous changes in character state observed among each country as determined from the MCMC tree was then recorded and compared to the number of expected under the null hypothesis of entirely random mixing by repeating this analysis on 1000 randomized trees. A matrix of the observed-minus-expected character state changes was then constructed to determine the strength of migration (positive values) or population substitution (negative values). All these analyses were conducted using the PAUP* package (Wilgenbusch & Swofford., 2003).

3.1.3 Results

3.1.3.1 Phylogeography of Africa 2 RABV

The MCC tree of 97 complete N sequences is shown in Figure 7. The topology was similar to those of earlier phylogenetic analyses of the N gene (Durr *et al.*, 2008, Kissi *et al.*, 1995). The sequences were clearly divided into two major clusters, identified as the 'Cosmopolitan' and 'Africa 2'. The Cosmopolitan clade includes isolates, which are distributed in north, central, east, and the south of Africa as previously shown (Bourhy *et al.*, 2008, Kissi *et al.*, 1995, Nel & Markotter., 2007). This study provides the first evidence for a widely distributed Africa 2 clade, comprising dog isolates that have a large geographic range in West and Central Africa, including Guinea, Sierra Leone, Senegal, Niger, Nigeria, Mauritania, Ivory Coast, Burkina Faso, Cameroon, Benin, Chad, Mali, Gambia and Republic of Central Africa (RCA) (Annex 7).

An additional phylogenetic analysis was performed on a larger data set of 134 sequences comprising only sequences belonging to the Africa 2 clade. This clade has a large distribution in West and Central Africa with only very little overlap with the Africa 1 clade in RCA and Nigeria. The Africa 2 sequences could also be arbitrarily grouped into 8 main groups (A to H) on the basis of phylogenetic placement, all supported by strong Bayesian posterior probability values (Figure 8 and Annex 7). Groups A and D, consisted of one isolate each, from Chad (Chad 73/2006) and Benin (8697BEN), respectively. Group B included isolates from Guinea, Sierra

Leone, Gambia and southern Senegal (isolate Nb 07023SEN). Group C consisted of one isolate originating in Chad (isolate 9218TCH), and another from the Northwestern part of RCA (isolate Nb 07149RCA). Group E contained viruses sampled from Cameroon, Chad, Nigeria, and one isolate from Niger dated 1990 (9014NIG). Group F included isolates originating in Niger and Burkina, while group G consisted of viruses from Mauritania, Burkina Faso, and Senegal. Group H contained viruses from Mauritania, Mali, Burkina Faso, and Ivory Coast.

3.1.3.2 The time-scale of RABV evolution

The mean rate of nucleotide substitution estimated for the N gene of isolates belonging to the Africa 2 clade using a Bayesian MCMC approach was 3.82×10^{-4} subs/site/year (95% HPD = $2.62 - 5.02 \times 10^{-4}$ subs/site/year). These rates are strongly consistent with previous analyses of substitution rates in fox RABV in Europe and mongoose RABV in Africa (Bourhy *et al.*, 1999, Davis *et al.*, 2007), as well as a more global analysis of dog RABV sampled worldwide (Bourhy *et al.*, 2008). Using the same approach, the TMRCA of the Africa 2 clade was estimated. The average age of the genetic diversity in this clade is 163 years (95% HPD = 72 – 288 years), which corresponds to the year 1845 (1936 – 1720) (Figure 8).

Figure 7. MCC tree of 97 sequences estimated from the N gene of RABV. Horizontal branches are drawn to a scale of estimated year of divergence (coalescence), with tip times reflecting sampling date (year). Posterior probabilities values (>90%) are shown for key nodes.

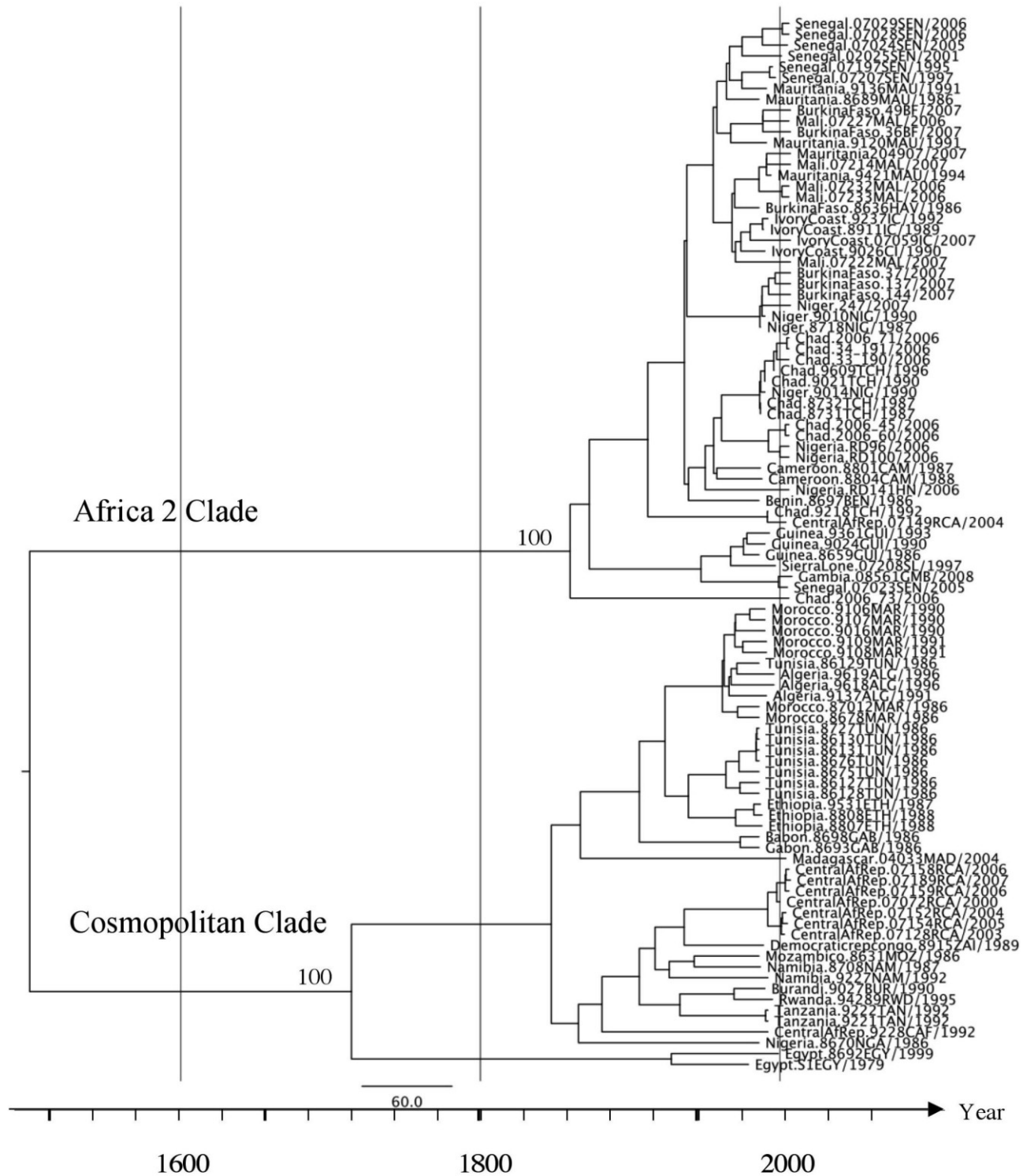
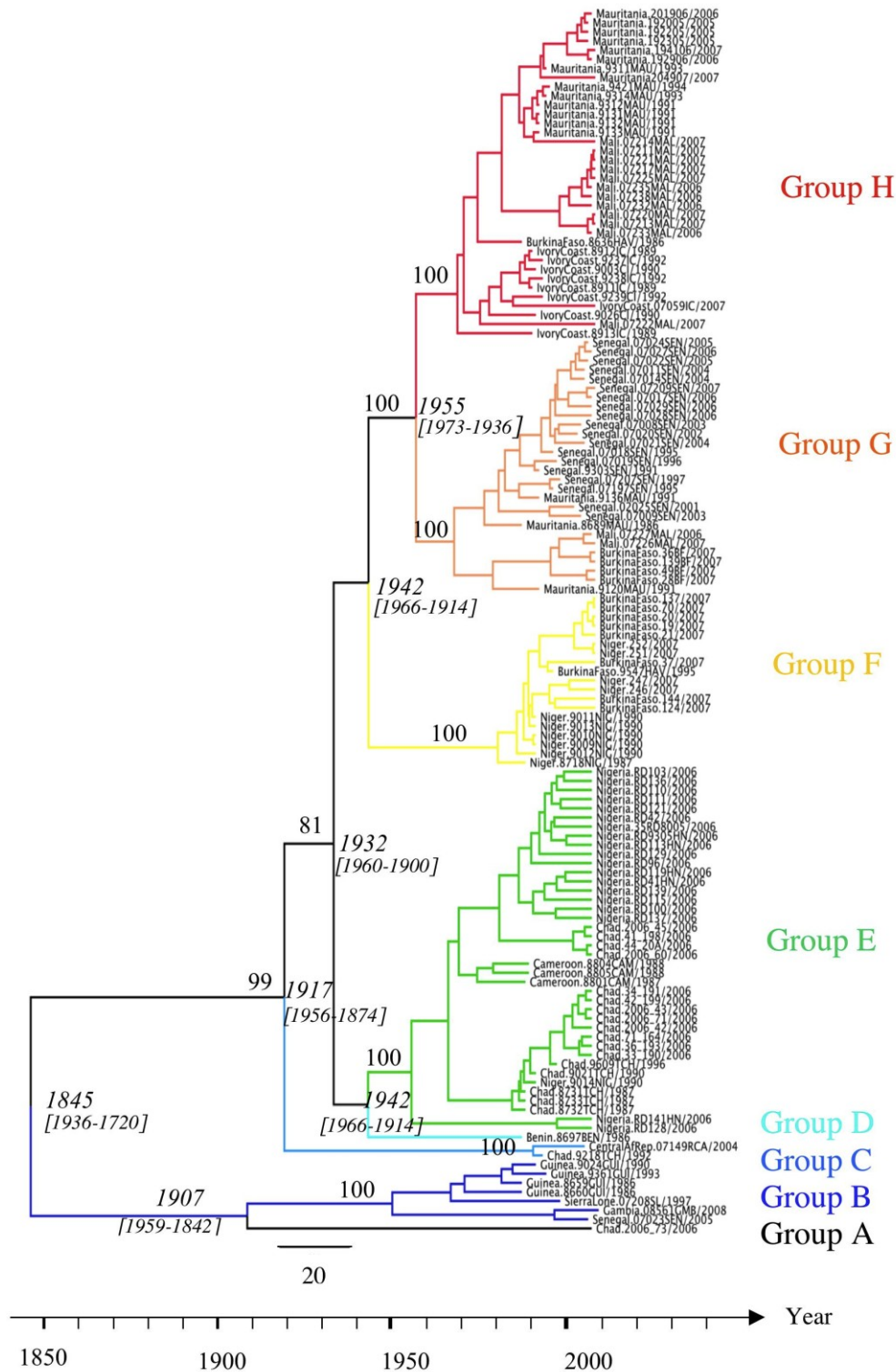


Figure 8. MCC tree of 134 sequences of N gene of the Africa 2 clade. The TMRCA are indicated for the clade and the major groups identified in the study.



3.1.3.3 Population and spatial dynamics of dog RABV

As the analysis of the time-scale of RABV evolution indicated that the colonization of this virus in Africa occurred relatively rapidly, the dynamics of this spatial diffusion were investigated in more detail. Overall, this analysis revealed a very strong population subdivision ($p \leq 0.001$), similar to that observed for RABV in other geographical areas (Bourhy *et al.*, 2008). Indeed, of 182 pairwise comparisons, only 11 (6%) exhibited a positive correlation between countries (> 0), indicative of migration between them. The strongest evidence for inter-country migration are between Chad-to-Nigeria (1.06), Chad-to-Benin (0.903), Chad-to-RCA (0.929), Chad-to-Cameroon (0.777), Chad-to-Niger (0.333), Niger-to-Burkina Faso (0.813), Burkina Faso-to-Mali (0.636) indicative of a general westward movement across West and Central Africa. However, in all cases the strength of migration was weak, with population subdivision by far the strongest signal in these data.

3.1.4 Discussion

The results described here provide the first evidence for the spread of dog RABV in West and Central Africa, associated with the spread of an Africa 2 clade, the dominant lineage of RABV in this area. Notably, this clade is surrounded in the north, the east and the south by the cosmopolitan clade (Figure 7). Such a spatial and phylogenetic distinction strongly supports the idea that these clades represent independent introductions into Africa.

More notably, the recent TMRCA for this Africa 2 clade, with a mean date of 1845 (95% HPD = 1936 – 1720), is consistent with the documented dates of colonization in Africa, and particularly of the expanding French influence in West and Central Africa. In fact, the French had focused main colonisation efforts on the Senegal River area and its hinterland just at the beginning of the nineteenth century, establishing their control in 1880 over much of Northern, Western, and Central Africa. By the early years of the twentieth century the French held most of what would come to be their colonial territory in West and Central Africa (including present day Mauritania, Senegal, Mali, Burkina Faso, Benin, Guinea, Côte d'Ivoire, Chad, RCA, Niger and Republic of Congo).

The analysis of phylogeographic structure also provides information on the pattern of RABV spread in this part of Africa. In particular, the presence of sequences sampled from Chad in both multiple clades and particularly those that tend to fall basal in tree, and that those sequences sampled from countries bordering the Atlantic Ocean tend to fall at distal locations, is suggestive of an initial introduction of RABV into the eastern part of Central-West Africa,

with Chad a potential source population, followed by a westward diffusion. However, the pattern is not strong and will need to be confirmed with a larger sample of sequences and spatial data.

The detailed phylogeographic analysis reveals that the Africa 2 clade spread rapidly to West and Central Africa. Indeed, it is possible that each clade of Africa 2 in its local dog population outcompetes the other RABV variants circulating in neighbouring localities, perhaps because of immunological exclusion, and that this reinforces the spatial subdivision we observe, although this will need to be tested on a larger sample of data.

The very strong geographical clustering of RABV, as well as a time-scale of spatial diffusion measured in decades, argues against any model in which superspreaders disseminate their virus rapidly over a large territory. Therefore, findings of this study sit in marked contrast with a recent study based on high-resolution temporal and spatial data which suggested that RABV spreads rapidly and continually from endemic rabies regions in Africa (Hampson *et al.*, 2007). Specifically, the authors explained the occurrence of rabies epidemics in southern and eastern Africa by suggesting that some 'superspreader' dogs may transmit the disease over large distances (Hampson *et al.*, 2007).

3.2 PHYLOGENETIC ANALYSES OF DOG RABIES VIRUSES FROM BURKINA FASO

De Benedictis P, Sow A, Fusaro A, Veggiato C, Talbi C, Kaboré A, Dundon WG, Bourhy H, Capua I. Phylogenetic analysis of rabies viruses from Burkina Faso, 2007. Zoonoses Public Health. 2010 Dec;57(7-8):e42-6.

3.2.1 Background

Previous molecular epidemiological studies of rabies in Africa (Bourhy *et al.*, 2008, David *et al.*, 2007, Kissi *et al.*, 1995) have revealed that at least four clades of RABV circulate in the dog population in the African continent.

The study described in chapter 3.1 has shown with a large panel of samples collected from West and Central Africa that Africa 2 RABVs circulate as a series of spatially distinct clusters that experience relatively little contact among them. The very strong geographical clustering of RABV sequences, as well as a time-scale of spatial diffusion measured in decades, argues against any model in which superspreaders disseminate their virus rapidly over a large territory (Hampson *et al.*, 2007).

From a genetic point of view, the Africa 2 clade has been arbitrarily grouped into 8 main groups (A to H). Interestingly, samples from Burkina Faso fell into two out of the 8 groups identified, namely group F and G (see paragraph 3.1.3.1). This finding warranted a special attention for further investigations and the need for enforced and coordinated National control programmes which would take advantages from the epidemiological data available from the field. The aim of this investigation was, therefore, to provide more detailed information on the genetic characteristics of RABVs obtained in Burkina Faso in 2007 and to further understand the geographical distribution and transboundary spread of this infection within the canine population.

3.2.2 Materials and methods

3.2.2.1 National survey and activities of the National Veterinary Laboratory

In Burkina Faso, an average of 3,800 cases of bites and scratches from domestic animals, mostly dogs, are recorded annually, resulting in over one hundred human fatal cases being officially reported annually (Coulibaly & Yameogo, 2000).

From 2001 to 2007, more than 3,500 brain samples, suspected of rabies infection, were submitted to the Laboratoire National d'Elevage (LNE) in Ouagadougou, the only Veterinary Institute in Burkina Faso for rabies diagnosis. Samples are routinely analyzed by the Fluorescent Antibody Test (FAT) (Anonymous, 2010) and brains confirmed as positive are usually archived at -20°C . Out of 101 rabies cases confirmed in 2007, 96 (95%) were dogs, of which 79 (82.3%) were reported as stray dogs (see Table 7). All of the submitted cases manifested clinical signs indicative of rabies infection, such as anxiety, abnormal behaviour, aggressiveness and sometimes paralysis, and had been humanely killed following an episode of aggression and biting of humans.

Table 7: Rabies suspected cases submitted to the Laboratoire National d'Elevage (LNE) during 2007. Of 3,500 suspected samples submitted to the LNE, 2,020 (57.7%) have been identified as rabies cases from 2001 to 2007.

Results	Species of origin				
	Rodent	Canine	Feline	Primate	Total
Positive	0	96	4	1	101
Negative	3	65	6	2	76
Total	3	161	10	3	177

3.2.2.2 Samples and sequencing

Thirty-two brains of dogs from different rabies cases in 2007 were selected for this study based on their geographical origin. The virus samples originated from 6 out of the 13 regions of Burkina Faso and are listed in Annex 8. After confirmation by FAT (Anonymous, 2010), brain samples were diluted in sterile phosphate buffer saline (PBS) in a ratio of 1:10. Viral RNA was extracted using the Nucleospin RNA II kit (Macherey-Nagel). RT-PCR was performed using the One Step RT-PCR kit (Qiagen), and sequences of the complete open reading frame (1350 nt) of the gene encoding the Nucleoprotein (N) were generated with specific primers and the Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems). Sequences were analyzed using a 3100 Avant Genetic Analyser (Applied Biosystems).

3.2.3 Phylogenetic analyses

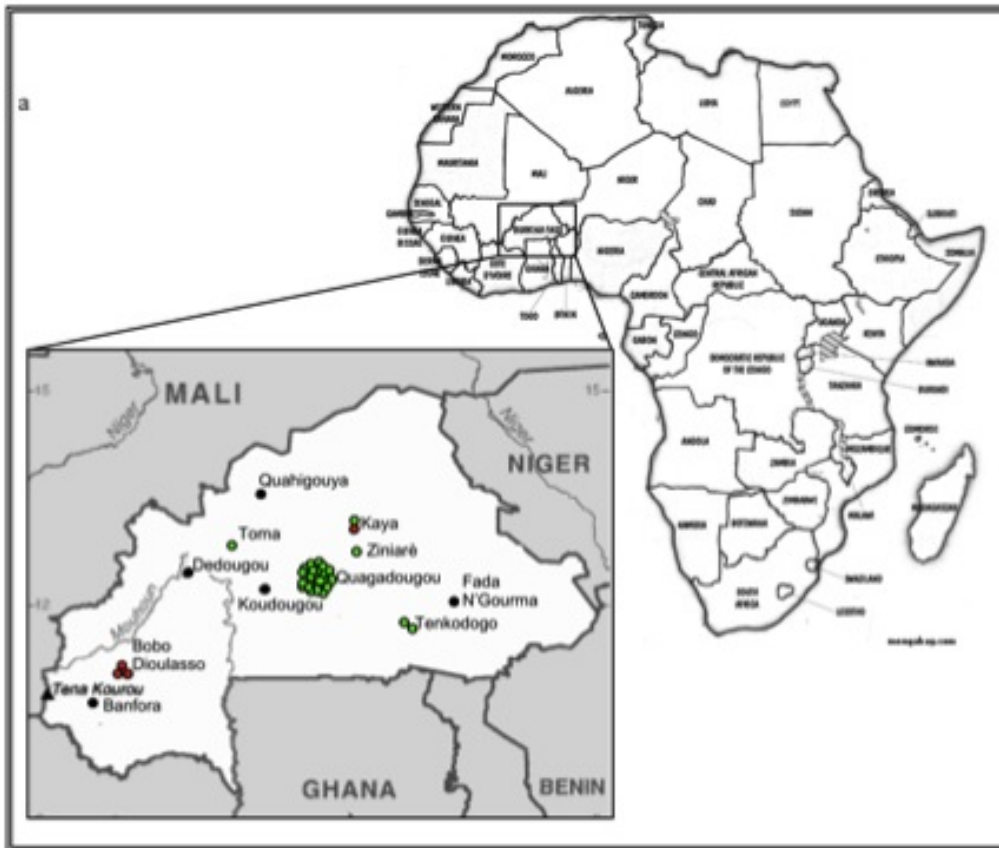
Phylogenetic analysis was performed using the neighbour-joining method with 1000 times bootstrapping, as implemented by the MEGA 4 program (Tamura *et al.*, 2007). Data obtained was subsequently confirmed by Bayesian methods. The best-fit model of nucleotide

substitution was identified, by using Akaike Information Criterion as implemented in ModelTest v3.7 (Posada & Crandall, 1998), as the general reversible GTR+I+ γ_4 model with the frequency of each substitution type, proportion of invariant site (I) and gamma distribution of among site rate variation with four rate categories (γ_4) estimated from the empirical data. Bayesian methods implemented with the computer program MrBayes program vers. 3.1.1 (Ronquist & Huelsenbeck, 2003) were used to obtain the final phylogeny and assess the statistical support for clades. Specifically, a Markov chain Monte Carlo search for one million generations was applied using two runs and four chains (temperature=0.05) and represented the results of the search as a 50% majority rule consensus tree. Clades with a Bayesian posterior probability (BPP) ≥ 0.95 were considered robust or strongly supported.

3.2.4 Results

As expected, phylogenetic analysis of the Burkina Faso RABVs revealed that all viruses tested belonged to the Africa 2 clade, which is distributed in the vast majority of Western Sub-Saharan countries and in Somaliland (see chapters 1.5. and 3.1). Close relatedness with RABVs from neighbouring countries, e.g. Niger, Mauritania, Benin, Cameroon, Chad and Guinea was observed (Annex 9). Two distinct well-supported groups (posterior probability over 97) within the study group were confirmed. The major group F included 28 out of the 32 viruses analyzed and clustered with RABVs from Niger (similarity ranged from 98.7% to 99.1%). The minor group G comprised four RABVs (28BF/2007, 36BF/2007, 49BF/2007, 139BF/2007) more closely related to a RABV 8689MAU from Mauritania (similarity ranging from 98.3 to 98.6%) than the other 28 RABVs from Burkina Faso. These four RABVs were collected from dogs from two distinct and distant geographical areas in the Southern-West and North-Central regions of Burkina Faso (Figure 9). All 32 viruses studied here showed low nucleotide sequence similarities (94.6-96%) with 8836HAV, a canine virus obtained in 1986 and the only sequence available from Burkina Faso in the database at the time of analysis. Analysis of the amino acid sequence revealed mostly synonymous mutations even for viruses of group G. The only amino acid changes identified were M78T, G413R, and R422M in 36BF/2007, 9BF/2007 and 142BF/2007 strains respectively.

Figure 9. Map of Burkina Faso, showing the origin of the RABVs studied. Green circles represent the sites where the group F viruses were collected, while red circles indicate the sites of origin of RABVs belonging to group G.



3.2.5 Discussion

The results of this investigation indicate that RABV belonging to the Africa 2 lineage were circulating in 6 out of the 13 regions of Burkina Faso in 2007. No other African lineage was identified during this period. A few non-synonymous mutations were identified among the strains sequenced. This observation merits further investigation given that the resulting amino acid changes are in positions that are normally highly conserved even between different RABV lineages (David *et al.*, 2007, Kissi *et al.*, 1995).

This study also confirms the circulation in Burkina Faso of two groups, namely F and G, distinct from a previously described RABV from 1986. Viruses within group F were very similar to contemporary and historical variants identified in Niger, suggesting a consolidated means for transboundary spread. Burkina Faso RABVs belonging to group G are mostly related to strains that are and have been circulating in Mauritania, but also, to a lesser extent to 88036HAV, the only available Burkina Faso virus from 1986. No further genetic groups, other than those previously identified in chapter 3.1, were found in this study.

Therefore, with reference to group G viruses currently circulating in Burkina Faso, transboundary spread from Mauritania through Mali or vice versa could be a possible origin of the viruses, although population subdivision cannot be excluded given the relatively small sample size. Transboundary spread within neighboring countries, with two parallel cycles of rabies currently identified in Burkina Faso, could be a possible origin of these viruses.

3.3 PHYLOGEOGRAPHY AND EVOLUTIONARY ANALYSES OF FOX RABIES CIRCULATING IN ITALY FROM 2008 TO 2011

Manuscript in preparation

3.3.1 Background

Wildlife rabies in Europe is caused by RABVs of the Cosmopolitan clade (Bourhy *et al.*, 1999). Two main reservoir species are known as responsible of disease maintenance in the European continent, namely the red fox (*Vulpes vulpes*) and the racoon dog (*Nyctereutes procyonoides*), the latter being mostly diffused in North-eastern Europe (Bourhy *et al.*, 1999). Ancestral roots of this virus in Europe date back to the 18th century before it spread worldwide by means of human-mediated dispersal and natural wildlife migration roots (Badrane & Tordo., 2001, Bourhy *et al.*, 1999, Smith *et al.*, 1992). A comprehensive phylogenetic analysis of European RABVs revealed a number of distinct groups, each associated to a geographical area, suggesting that it has spread westwards and southwards across Europe during the last century (Bourhy *et al.*, 1999). More recently a comprehensive molecular study tracing the evolutionary history of RABVs circulating in the Balkans using a large panel of data confirmed the previous RABV geographic pattern (McElhinney *et al.*, 2011). Findings from those studies revealed that the red fox is the responsible of the maintenance of several genetic lineages and that nomenclature may no longer adequately describes all their geographical distributions (Bourhy *et al.*, 1999, McElhinney *et al.*, 2011). As a matter of fact, at least 2 additional lineages have been recently described (McElhinney *et al.*, 2011) other than the previous identified Eastern, Central and Western European lineages (Bourhy *et al.*, 1999), highlighting the need for an updated common nomenclature for all European RABVs.

In Italy, the Northeastern territories have been affected by rabies in the 1970s and 1980s and more recently in the period from 1991 to 1995. Epidemics of rabies in foxes were linked to the epidemiological situation of infection in Austria and the nearby territories of former Yugoslavia, now Slovenia. For this reason, the risk of rabies re-introduction in Italy from the bordering areas has long been recognised. Vaccination campaigns using oral rabies vaccines have been conducted targeting the fox population in these areas in 1989 and between 1992 and 2004 (Mutinelli *et al.*, 2004). The last case of rabies was diagnosed in a fox in the province of Trieste on the border with Slovenia in December 1995, and Italy has been classified as rabies-free since 1997.

As for previous fox rabies epidemics, the recent outbreaks (2008-2011) have been linked to the epidemiological situation in the Balkan region. Re-emergence of rabies in Italy was retained thus a consequence of the westward spread of the infection front (De Benedictis *et al.*, 2008). Since its re-emergence in 2008, the fox rabies epidemic in North-eastern Italy spread westwards extending a radius of around 124 kilometres. In November 2009 the epidemic reached the province of Belluno (Veneto region) which has been the most affected area with a total of 216 detected animal rabies cases, out of which 81.9% (177/216) were confirmed in foxes, as of August 2011.

In this study, the phylogeny and evolution of RABVs circulating in North-eastern Italy have been investigated in depth, with the final aim of offering a comprehensive picture of the recent epidemic dynamics, constraints and tools for control improvements.

3.3.2 Materials and methods

3.3.2.1 Viruses and sequencing

In order to investigate the genetic diversity of RABV circulating in North-eastern Italy, 113 sequences, 600 nt in length, of the partial N gene obtained from fox samples were selected out of all brain specimens (n= 287) collected during the 2008-2011 epidemic in North-eastern Italy. Additional sequencing of the entire G gene (1572 nt) and the intergenic region G-L (520 nt) was performed for 128 fox brain specimens selected within the total 287. For all samples, the date of sampling and a precise spatial co-ordinate were available. Relevant epidemiological information for all those RABV isolates analysed in this study is presented in Appendices 4 and 10.

Viral RNA was extracted from clinical samples by using the Nucleospin RNA II kit, according to the manufacturer's instructions (Macherey-Nagel, Germany). Briefly, one hundred microlitres of sample suspension were used for the extraction, and RNA was eluted in a final volume of 60 μ l and stored at -80 $^{\circ}$ C. RT-PCR and sequencing reactions were performed, by using primers listed in Annex 11.

3.3.2.2 Phylogenetic analysis

Maximum-likelihood (ML) trees were estimated using the best-fit general time-reversible (GTR) model of nucleotide substitution with gamma-distributed rate variation among sites, and a heuristic SPR branch-swapping search available in PhyML version 3.0 (Guindon &

Gascuel., 2003). Parameter values for the GTR substitution matrix, base composition, gamma distribution of the rate variation among sites (with four rate categories, Γ_4), and proportion of invariant sites (I) were estimated directly from the data using MODELTEST (Posada & Crandall., 1998). A bootstrap resampling process (1,000 replications) using the neighbor-joining (NJ) method was used to assess the robustness of individual nodes of the phylogeny, incorporating the ML substitution model defined above.

3.3.2.3 Substitution rates and times to common ancestry

Rates of nucleotide substitution per site per year and the time to the most recent common ancestor (TMRCA) of the sampled data were estimated using the BEAST program, version 1.6.1 (Drummond & Rambaut., 2007), which employs a Bayesian Markov chain Monte Carlo (MCMC) approach. For each analysis, a flexible Bayesian skyline coalescent tree prior (Drummond *et al.*, 2005) and a codon based SRD06 model (Shapiro *et al.*, 2006) were used. Two molecular clock models — strict (constant) and uncorrelated lognormal relaxed clock — were compared by analysing values of the coefficient of variation (CoV) in Tracer (Drummond & Rambaut., 2007), such that CoV values of >0 are evidence of non-clock-like evolutionary behaviour. For both genes the lower 95% HPDs of CoV values of the relaxed molecular clock were approximately 0, so a strict molecular clock model was used to estimate the evolutionary dynamics. In all cases, uncertainty in the data is reflected in the 95% highest probability density (HPD) values for each parameter estimate, and in each case, chain lengths were run for sufficient time to achieve coverage as assessed using the Tracer v1.5 program (Drummond & Rambaut., 2007).

3.3.2.4 Phylogeography of RABV in Italy

To determine the spatial dissemination of the RABVs in Italy and Western Balkans and Eastern Europe, the sequences of the N gene were grouped into 7 geographic regions, namely Italy-A (Friuli Venezia Giulia, FVG), Italy-B (mountainous areas in the Veneto regions and the autonomous provinces of Trento e Bolzano) Italy-C (piedmont areas in the Veneto region and the autonomous province of Trento), Italy-D (strains collected during the 90' epidemic in FVG), Bosnia-Herzegovina, Croatia, Slovenia. Phylogenetically divergent sequences from other geographic regions were excluded. This resulted in a final data set of 160 viruses that could be used in the detailed spatial analysis.

Posterior distributions under the Bayesian phylogeographic model (Lemey *et al.*, 2009) were estimated using a MCMC method implemented in BEAST. The model incorporated the date of

sampling and used a strict molecular clock, BSP prior, and the SRD06 model of nucleotide substitution. All parameters reached convergence, as assessed visually using Tracer v 1.5. A maximum clade credibility (MCC) phylogenetic tree was estimated from the posterior distribution of trees generated by BEAST using the program TreeAnnotator v1.6.1 (Drummond & Rambaut., 2007) after the removal of an appropriate burn in (10% of the samples). The MCC tree was visualized using the program FigTree v1.3.1 (<http://tree.bio.ed.ac.uk/software/figtree/>).

3.3.3 Results

3.3.3.1 Phylogenetic analysis of fox RABVs in North-eastern Italy reveals the circulation of two viral populations

To investigate the genetic diversity of RABVs circulating in North-eastern Italy, the G gene and the N gene of respectively 128 and 113 Italian strains under study were aligned and compared with all the related sequences of viruses from Western Balkans and Eastern Europe available in GenBank (132 for the N gene and 26 for the G gene). Phylogenetic relationships between the Italian RABVs and representative sequences from Western Balkans were determined. The topologies of ML phylogenetic trees revealed substantial genetic diversity, exemplified as at least two distinct RABV introduction events and circulation in North-eastern Italy.

As expected, all RABVs circulating in North-eastern Italy from 2008 to 2011 cluster within the West European lineage and are closely related to strains collected from Western Balkans.

Analysis of the N and G phylogenies of the Italian fox samples identifies two distinct genetic groups of RABVs circulating in Italy, designated Italy-1 and Italy-2 (Figure 10). More specifically, the group Italy-1 includes all samples collected in FVG but one, which clusters within the second group, Italy-2, including all the sequences of fox samples collected in Veneto and the autonomous provinces of Trento and Bolzano (Figure 10). The only sequence from FVG in the clade Italy-2 was collected in December 2008, at the beginning of the epidemic and in a municipality close to that of the first case notified.

Phylogenetic analysis of the N gene showed that viruses belonging to Italy-1 group clustered with viruses from Croatia (2008 to 2010) Bosnia-Herzegovina (2006) and Slovenia (2002 to 2010), while Italy-2 samples were close related with viruses collected in Bosnia-Herzegovina (1986-2006), Croatia (2009 to 2010), Slovenia (2000) and Formerly Republic of Yugoslavia

(1986), indicating that the two distinct groups have been circulated in the Balkans before their introduction in Italy.

Unfortunately, for the G gene only sequences of viruses from Slovenia were available in GenBank.

Figure 10. ML tree of sequences estimated from the N gene of RABV.



3.3.3.2 The time-scale of RABV evolution

The mean rate of nucleotide substitution estimated for the Italian isolates using a Bayesian MCMC approach was 5.95×10^{-4} subs/site/year (95% HPD = $1.50 - 11.16 \times 10^{-4}$ subs/site/year) for the N gene and 4.38×10^{-4} subs/site/year (95% HPD = $2.57 - 6.39 \times 10^{-4}$ subs/site/year) for the G gene and the intergenic region G-L. These rates are consistent with previous analyses of substitution rates in fox RABV in Europe and mongoose RABV in Africa (Bourhy *et al.*, 1999) (Kuzmin *et al.*, 2008) (Davis *et al.*, 2007) (Van Zyl *et al.*, 2010), as well as a more global analysis of dog RABV sampled worldwide (Bourhy *et al.*, 2008).

Using the same approach, we were able to estimate the TMRCA of the Italian fox RABVs. The TMRCAs calculated for the N and G gene were respectively June 1993 (95% HPD, May 1984-October 2001) and November 2004 (December 1998-June 2008).

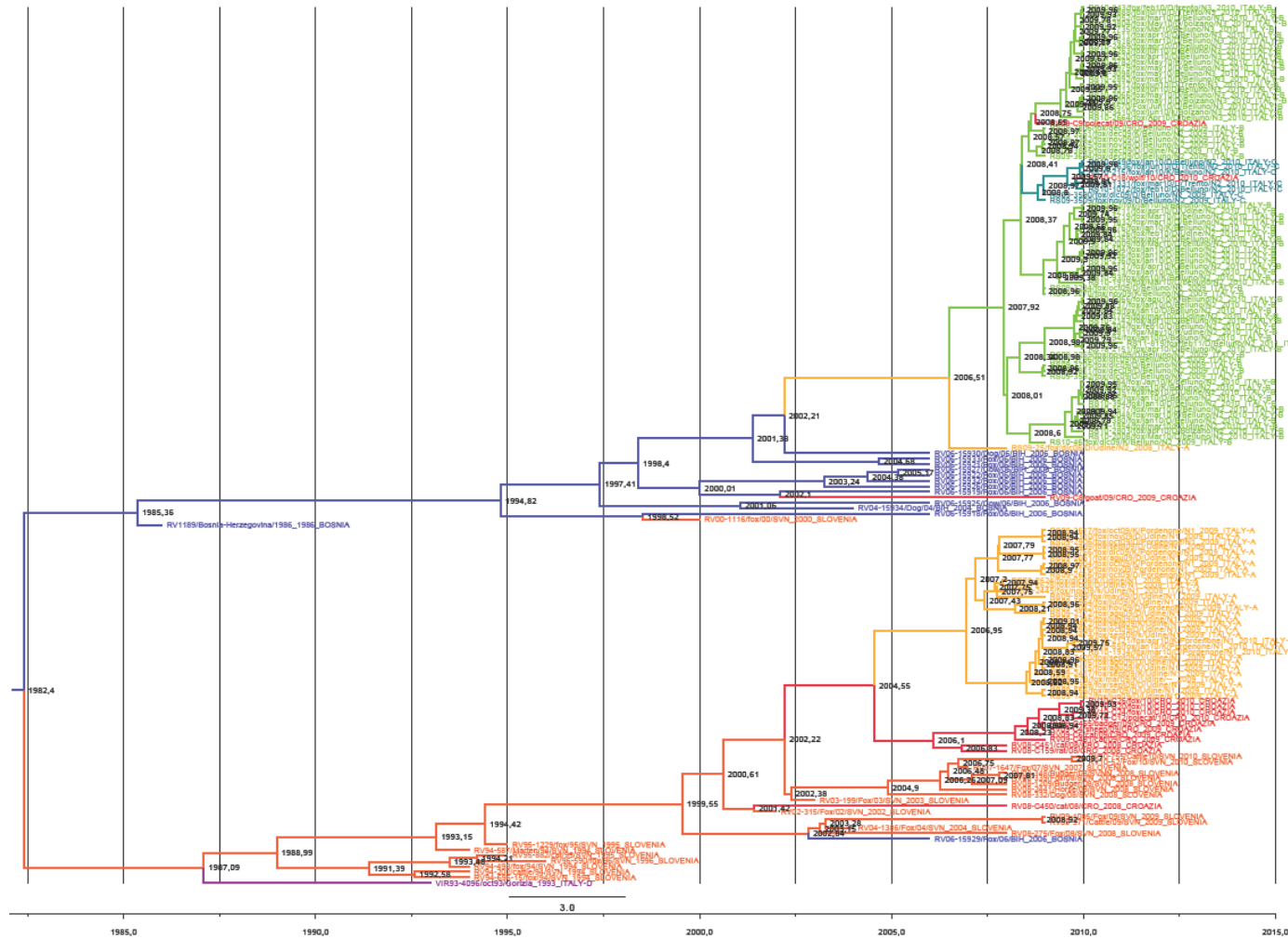
3.3.3.3 Italian RABVs originated in West Balkans

Phylogenetic analysis revealed that RABVs circulating in Italy clustered with viruses from Western Balkans (notably Bosnia-Herzegovina, Croatia and Slovenia), suggesting an extensive viral circulation in wildlife population across the borders in this geographical area.

To examine the extent and pattern of geographical structure in these data in a more quantitatively rigorous manner, a Bayesian phylogeny for the N gene was inferred, in which each RABV was assigned to a different geographic areas.

Two major clades were distinguishable in the maximum clade credibility (MCC) tree (see figure 11). Both of them included samples collected from Italy, Bosnia-Herzegovina, Croatia and Slovenia. More specifically, the MCC tree annotated with most probable nodal locations confirmed the existence of two distinct introductions of both Italy-1 and Italy-2 groups in Friuli Venezia Giulia, with viral migration of Italy-2 group toward western areas of Northern Italy. The likely ancestor strains for groups Italy-1 and Italy-2 come from Croatia and Bosnia-Herzegovina, respectively, with a plausible westwards spreading, although back migration events seem to occur, as i.e. to Croatia within the Italy-2 clade.

Figure 11. Maximum clade credibility (MCC) tree inferred for N gene sequences from Italy, Bosnia-Herzegovina, Croatia and Slovenia. Sequences are coloured according to the geographical origin. Yellow: Italy A. Bright green: Italy B Deep green: Italy C Purple:Italy D. Blue: Bosnia-Herzegovina. Red: Croatia. Orange: Slovenia.



3.3.3.4 Discussion

This investigation provides a comprehensive molecular epidemiological analysis of the fox RABV epidemic occurring in Italy from 2008 to 2011. A wide dataset and information availability from the field surveillance make this study almost unique, although it focuses on a limited time length and a small geographic area.

Viruses responsible of the recent 2008-2011 epidemic fall into the West European lineage of the RABV Cosmopolitan clade. Two genetic groups have been identified in Italy in the recent 2008-2011 epidemic: one mainly spread westwards to Veneto and one circulating in Friuli Venezia Giulia. Those genetic different viral populations have likely been introduced with a short delay, as supported by MCC results, and circulated occupying two distinct geographic areas. Viruses of both clades were found at the beginning of the 2008 epidemic in close municipalities. However, from December 2008, viruses of the so-called clade Italy-2 reappeared 10 months later and over 100 kms westwards. One possible explanation for this finding is that rabid foxes had probably crossed the northern territories of FVG unnoticed and spread westwards to the Veneto region. During 2009, official data collected at regional level indicated that the disease has been spreading to the FVG region mainly southwards (see Annex 3). Oral rabies vaccination (ORV) campaigns were implemented at the regional level, covering limited areas of the FVG region. Northern territories were left unvaccinated taking into account previous experience of fox rabies epidemics (Mutinelli *et al.*, 2004) and assuming that foxes would settle with difficulty into a mountain area in winter. Thus, ORV undertaken in 2009 in a limited area was able to control the disease only partially and by chance, in those areas where strains of the clade Italy-1 were circulating. Italy-1 viruses remained confined to FVG and no westwards spreading occurred.

The analysis of phylogeographic structure also provides information on the pattern of those two RABV clades. Bayesian MCCM analysis identified different MRCA originating in Western Balkans (Formerly Republic of Yugoslavia) for both clades, supporting the hypothesis of the lineage circulation across the region comprised between Bosnia-Herzegovina, Croatia, Slovenia and Italy, likely throughout fox population dynamics. In particular, viruses of the clade Italy-2 are separated from a Bosnian MRCA by a relatively long branch of about 4 years. Whether the virus circulated in the fox population within the West Balkan area and to what extent is still unclear with data publicly available to date. Four years are a sufficient period of time for the virus to move through Croatia and Slovenia, where rabies cases were in fact notified. Thus, transient decedents along this branch in these countries might just have not sampled or

sequenced. Indeed, there are very few isolates from Croatia and Slovenia sampled between 2002 and 2006.

This study uncovers the dynamics and mechanisms of viral evolution and transmission of RABVs in North-eastern Italy and will contribute to future control programmes at both national and regional level. A better ecological picture of the fox population in the pre-Alpine areas of Italy and in neighbouring areas, as well as of the effects of concomitant epidemics (Monne *et al.*, 2011), would certainly add crucial information on the dynamics of rabies re-emergence, distances covered by the infection and viral geographical segregations. With this purpose, genetic typing of hosts could help explaining the movement patterns of foxes and eventually confirming hypotheses inferred by phylogeography.

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ANNEXES

Annex 1. GenBank accession numbers of sequences used for primer set design (RABV = Rabies virus; LBV = Lagos bat virus; MOKV = Mokola virus; DUVV = Duvenhage virus; EBLV-1 = European bat lyssavirus type 1; EBLV-2 = European bat lyssavirus type 2; ABLV = Australian bat virus; KHUV = Khujand virus; ARAV = Aravan virus; IRKV = Irkut virus; WCBV = West Caucasian bat virus; SHIBV = Shimoni bat virus).

GenBank accession numbers				
RABV	FJ959397	EF206707	AB085828	EBLV-2
U03769	EU643590	EU293121	U27221	NC009528
EF564174	FJ866836	EU293113	EF206712	EF157977
U03770	FJ866835	EU293111	EF206711	EU293114
U03768	EU877071	EU549783	EF206710	ABLV
AY956319	EU877070	EU293115	LBV	NC003243
U27221	EU877069	EF437215	EU293108	AF081020
AB085828	EU877068	EU293116	EU259198	KHUV
U27220	EU877067	AB044824	EU293110	EF614261
U27219	FJ712196	AB085828	MOKV	ARAV
AB010494	FJ712195	AB009663	EU293118	EF614259
L20673	FJ712194	AY956319	EU293117	IRKV
EF206717	FJ712193	EU182346	D13767	EF614260
AB044824	AB128149	AY705373	NC006429	WCBV
AB128149	EU311738	AB010494	DUVV	EF614258
EF437215	NC001542	U03770	EU293119	SHIBV
EF206710	EF206720	U03769	EU293120	GU170201
EF206717	EF206719	U03768	EBLV-1	
EF206710	EF206718	EF206708	EU2931.09	
EF564174	EF206717	EF564174	EU293112	
EF437215	EF206716	DQ286762	EU626552	
AB128149	EF206715	U27219	EF157976	
AB044824	EF206714	U27220	EU626551	
AY956319	EF206713	EF206709	NC009527	

Annex 2. Field samples tested positive by the One Step RT-PCR and typed using pyrosequencing. Each sample was previously tested positive by FAT as a standard method whenever applicable, or by a PCR method previously described (Dacheux *et al.*, 2008). All samples were also analysed and typed by pyrosequencing, and confirmation was performed using Sanger sequencing results. Taxonomic identification at the species level matched perfectly with Sanger sequencing results for all tested samples. (1): skin biopsy of upper lips. (2): Salivary glands. * : brain specimen obtained from mouse infected intracerebrally. ** : skin biopsy obtained from mouse infected intracerebrally, ***: same animal.

Identification of samples tested					Sample tested by RT-hnPCR (detection results)
Virus species	Strain	Host/vector species	Source of sample	Continent/ Country of origin	
			(if different from host/vector species)		
RABV	Europe				
	3833/93	Fox (<i>Vulpes vulpes</i>)		Italy	
	4142/93	Fox (<i>Vulpes vulpes</i>)		Italy	
	4316/93	Fox (<i>Vulpes vulpes</i>)		Italy	
	629/93	Cat (<i>Felis catus</i>)		Italy	
	1437/93	Fox (<i>Vulpes vulpes</i>)		Italy	
	4488/93	Fox (<i>Vulpes vulpes</i>)		Italy	
	1861/93	Fox (<i>Vulpes vulpes</i>)		Italy	
	571/93	Fox (<i>Vulpes vulpes</i>)		Italy	
	2020/93	Fox (<i>Vulpes vulpes</i>)		Italy	
	13/94	Fox (<i>Vulpes vulpes</i>)		Italy	
	558/93	Badger (<i>Meles meles</i>)		Italy	
	2665/93	Fox (<i>Vulpes vulpes</i>)		Italy	
	472/93	Fox (<i>Vulpes vulpes</i>)		Italy	
	4096/93	Fox (<i>Vulpes vulpes</i>)		Italy	
	834/93	Fox (<i>Vulpes vulpes</i>)		Italy	
	3988/93	Fox (<i>Vulpes vulpes</i>)		Italy	
	4055/93	Fox (<i>Vulpes vulpes</i>)		Italy	
	599/93	Fox (<i>Vulpes vulpes</i>)		Italy	
	3988/93	Fox (<i>Vulpes vulpes</i>)		Italy	
	3825/93	Fox (<i>Vulpes vulpes</i>)		Italy	
	600/93	Fox (<i>Vulpes vulpes</i>)		Italy	
	25/09RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	2420/09RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	1459/09RS	Dog (<i>Canis lupus familiaris</i>)		Italy	

	134/09RS	Badger (<i>Meles meles</i>)		Italy	
	3433/09RS	Donkey (<i>Equus asinus</i>)		Italy	
	3704/09RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	209/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	213/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	215/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	223/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	231/10RS	Badger (<i>Meles meles</i>)		Italy	
	236/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	237/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	309/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	310/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	378/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	380/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	383/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	388/10RS	Roe deer (<i>Capreolus capreolus</i>)		Italy	
	389/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	394/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	395/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	396/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	400/10RS	Cat (<i>Felis catus</i>)		Italy	
	401/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	409/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	410/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	414/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	476/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	Yes (positive)
	480/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	484/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	486/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	488/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	542/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	549/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	555/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	556/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	565/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	688/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	693/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	694/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	695/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	856/10RS	Roe deer (<i>Capreolus</i>)		Italy	

		<i>capreolus</i>)			
	867/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	1015/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	1016/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	1017/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	1018/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	1072/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	1073/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	1074/10RS	Badger (<i>Meles meles</i>)		Italy	
	1075/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	555/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	556/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	557/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	Yes (positive)
	702/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	704/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	705/10RS	Cat (<i>Felis catus</i>)		Italy	
	715/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	721/10RS	Horse (<i>Equus caballus</i>)		Italy	
	788/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	1117/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	1126/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	1251/10RS	Roe deer (<i>Capreolus capreolus</i>)		Italy	
	1252/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	1331/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	1334/10RS	Stone marten (<i>Martes foina</i>)		Italy	
	1351/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	1389/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	1390/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	1397/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	1400/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	1402/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	1493/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	1494/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	1496/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	1498/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	1612/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	1617/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	1618/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	1619/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	

1513/10RS	Cat (<i>Felis catus</i>)		Italy	
1514/10RS	Cat (<i>Felis catus</i>)		Italy	
1684/10RS	Roe deer (<i>Capreolus capreolus</i>) (		Italy	
1585/10RS	Roe deer (<i>Capreolus capreolus</i>)		Italy	
1709/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
1719/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
1735/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
1917/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
1918/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
1919/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
1920/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
1934/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
1954/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
2008/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
2009/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
2011/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
2012/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
1832/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
2014/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
1833/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
1891/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
1893/10SR	Fox (<i>Vulpes vulpes</i>)		Italy	
2121/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
2137/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
2141/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
2142/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
2151/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
2153/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
2227/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
2260/10RS	Roe deer (<i>Capreolus capreolus</i>)		Italy	
2261/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
2262/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
2263/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
2264/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
2265/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
2266/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
2269/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
2278/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
2339/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	

	2340/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	2373/10RS	Roe deer (<i>Capreolus capreolus</i>)		Italy	
	2432/10RS	Badger (<i>Meles meles</i>)		Italy	
	2433/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	2454/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	2469/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	2470/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	2471/10RS	Badger (<i>Meles meles</i>)		Italy	
	2490/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	2497/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	2511/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	2519/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	2520/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	2585/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	2602/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	2603/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	2604/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	2643/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	2644/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	2658/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	2659/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	2660/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	2664/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	2711/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	2778/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	2782/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	2788/10RS	Stone marten (<i>Martes foina</i>)		Italy	
	2803/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	2804/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	2811/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	2852/10RS	Stone marten (<i>Martes foina</i>)		Italy	
	2892/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	2979/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	3009/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	3065/10RS	Stone marten (<i>Martes foina</i>)		Italy	
	3066/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	3067/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	3068/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	3095/10RS	Prairie Dog (<i>Cynomys gunnisoni</i>)		Italy	

	3098/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	3200/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	3263/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	3311/10RS	Badger (<i>Meles meles</i>)		Italy	
	3313/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	3410/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	3417/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	3569/10RS	Badger (<i>Meles meles</i>)		Italy	
	3570/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	3636/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	3690/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	3729/10RS	Badger (<i>Meles meles</i>)		Italy	
	3888/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	4248/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	4449/10RS	Cat (<i>Felis catus</i>)		Italy	
	4457/10RS	Bovine (<i>Bos taurus</i>)		Italy	
	5031/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	5066/10RS	Fox (<i>Vulpes vulpes</i>)		Italy	
	6507/10RS	Badger (<i>Meles meles</i>)		Italy	
	6698/10RS***	Badger (<i>Meles meles</i>)		Italy	
	7171-1/10RS***	Badger (<i>Meles meles</i>)		Italy	Yes (positive)
	7171-2/10RS***	Badger (<i>Meles meles</i>)		Italy	Yes (negative)
	7171-3/10RS***	Badger (<i>Meles meles</i>)		Italy	Yes (positive)
	7171-4/10RS***	Badger (<i>Meles meles</i>)		Italy	
	RT2006/I	Racoon Dog (<i>Nyctereutes procyonoides</i>)	Mouse* (<i>Mus musculus</i>)	Latvia	
	RT2006/A	Fox (<i>Vulpes vulpes</i>)	Mouse* (<i>Mus musculus</i>)	France	
	RT2006/C	Fox (<i>Vulpes vulpes</i>)	Mouse* (<i>Mus musculus</i>)	France	
	RT2009/4	Fox (<i>Vulpes vulpes</i>)	Mouse* (<i>Mus musculus</i>)	France	
	Racoon Dog (Canis lupus familiaris)	Racoon Dog (<i>Nyctereutes procyonoides</i>)	Mouse* (<i>Mus musculus</i>)	Poland	
	RT2009/7	Racoon Dog (<i>Nyctereutes procyonoides</i>)	Mouse* (<i>Mus musculus</i>)	Latvia	
	93127FRA	Fixed strain	Mouse* (<i>Mus musculus</i>)	France	
	GS7 1-11	Fox (<i>Vulpes vulpes</i>)	Mouse* (<i>Mus musculus</i>)	France	
	ST2006/A (GS9)	Fox (<i>Vulpes vulpes</i>)	Mouse* (<i>Mus musculus</i>)	France	

2195PV1/2006	Dog (<i>Canis lupus familiaris</i>)		Lithuania	
2341V1/2006	Raccoon Dog (<i>Nyctereutes procyonoides</i>)		Lithuania	
7212PV1/2007	Marten (<i>Martes foina</i>)		Lithuania	
11243PV1/2007	PoleCat (<i>Felis catus</i>)		Lithuania	
4266PV1/2006	Marten (<i>Martes foina</i>)		Lithuania	
4400PV1/2006	Marten (<i>Martes foina</i>)		Lithuania	
5298PV1/2006	Dog (<i>Canis lupus familiaris</i>)		Lithuania	
5485PV1/2006	Marten (<i>Martes foina</i>)		Lithuania	
3569PV1/2006	Raccoon Dog (<i>Nyctereutes procyonoides</i>)		Lithuania	
6718V1/2006	Marten (<i>Martes foina</i>)		Lithuania	
6608V1/2006	Fox (<i>Vulpes vulpes</i>)		Lithuania	
6722V1/2006	Fox (<i>Vulpes vulpes</i>)		Lithuania	
95V1/2006	Raccoon Dog (<i>Nyctereutes procyonoides</i>)		Lithuania	
204PV1/2006	Fox (<i>Vulpes vulpes</i>)		Lithuania	
228PV1/2006	Raccoon Dog (<i>Nyctereutes procyonoides</i>)		Lithuania	
358V1/2006	Raccoon Dog (<i>Nyctereutes procyonoides</i>)		Lithuania	
362PV1/2006	Raccoon Dog (<i>Nyctereutes procyonoides</i>)		Lithuania	
402PV1/2006	Fox (<i>Vulpes vulpes</i>)		Lithuania	
455V1/2006	Fox (<i>Vulpes vulpes</i>)		Lithuania	
576PV1/2006	Raccoon Dog (<i>Nyctereutes procyonoides</i>)		Lithuania	
642PV1/2006	Raccoon Dog (<i>Nyctereutes procyonoides</i>)		Lithuania	
686PV1/2006	Raccoon Dog (<i>Nyctereutes procyonoides</i>)		Lithuania	
778V1/2006	Dog (<i>Canis lupus familiaris</i>)		Lithuania	
807V1/2006	Raccoon Dog (<i>Nyctereutes procyonoides</i>)		Lithuania	
18747V1/2006	Raccoon Dog (<i>Nyctereutes</i>)		Lithuania	

		<i>procyonoides</i>)			
18697V1/2006		Fox (<i>Vulpes vulpes</i>)		Lithuania	
5964PV1/2006		Fox (<i>Vulpes vulpes</i>)		Lithuania	
5955PV1/2006		Fox (<i>Vulpes vulpes</i>)		Lithuania	
6612V1/2006		Fox (<i>Vulpes vulpes</i>)		Lithuania	
5960PV1/2006		Raccoon Dog (<i>Nyctereutes procyonoides</i>)		Lithuania	
6605V1/2006		Raccoon Dog (<i>Nyctereutes procyonoides</i>)		Lithuania	
6641PV1/2006		Fox (<i>Vulpes vulpes</i>)		Lithuania	
6720V1/2006		Fox (<i>Vulpes vulpes</i>)		Lithuania	
6374PV1/2006		Raccoon Dog (<i>Nyctereutes procyonoides</i>)		Lithuania	
6091PV1/2006		Fox (<i>Vulpes vulpes</i>)		Lithuania	
6128V1/2006		Raccoon Dog (<i>Nyctereutes procyonoides</i>)		Lithuania	
6345PV1/2006		Raccoon Dog (<i>Nyctereutes procyonoides</i>)		Lithuania	
6127V1/2006		Fox (<i>Vulpes vulpes</i>)		Lithuania	
<i>Africa</i>					
2BF/2007		Dog (<i>Canis lupus familiaris</i>)		Burkina Faso	
4BF/2007		Dog (<i>Canis lupus familiaris</i>)		Burkina Faso	
8BF/2007		Dog (<i>Canis lupus familiaris</i>)		Burkina Faso	
9BF/2007		Dog (<i>Canis lupus familiaris</i>)		Burkina Faso	
10BF/2007		Dog (<i>Canis lupus familiaris</i>)		Burkina Faso	
12BF/2007		Dog (<i>Canis lupus familiaris</i>)		Burkina Faso	
18BF/2007		Dog (<i>Canis lupus familiaris</i>)		Burkina Faso	
28BF/2007		Dog (<i>Canis lupus familiaris</i>)		Burkina Faso	
31BF/2007		Dog (<i>Canis lupus familiaris</i>)		Burkina Faso	
33BF/2007		Dog (<i>Canis lupus familiaris</i>)		Burkina Faso	
36BF/2007		Dog (<i>Canis lupus familiaris</i>)		Burkina Faso	
41BF/2007		Dog (<i>Canis lupus familiaris</i>)		Burkina Faso	
45BF/2007		Dog (<i>Canis lupus familiaris</i>)		Burkina Faso	

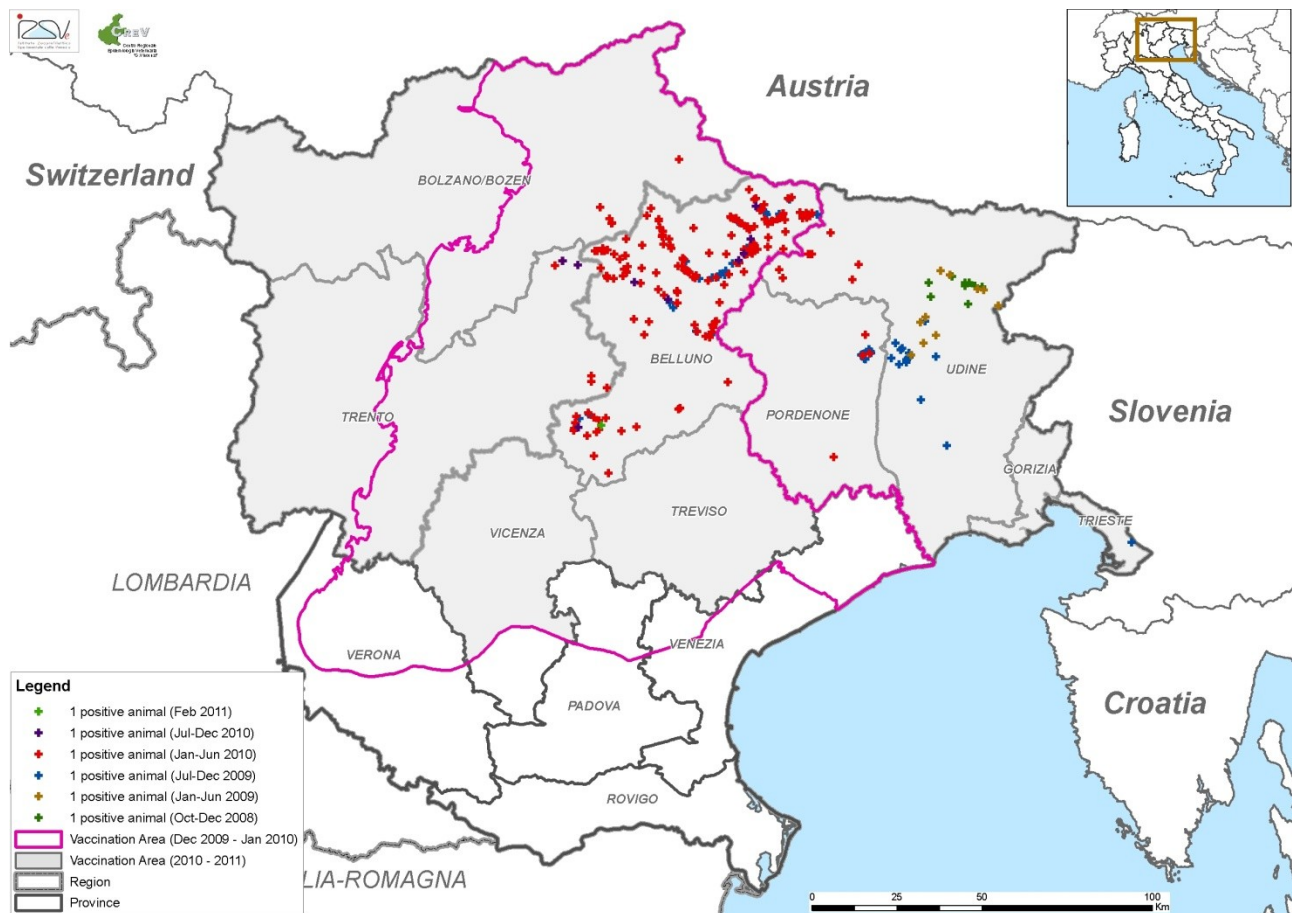
	46BF/2007	Dog (<i>Canis lupus familiaris</i>)		Burkina Faso	
	49BF/2007	Dog (<i>Canis lupus familiaris</i>)		Burkina Faso	
	57BF/2007	Dog (<i>Canis lupus familiaris</i>)		Burkina Faso	
	69BF/2007	Dog (<i>Canis lupus familiaris</i>)		Burkina Faso	
	82BF/2007	Dog (<i>Canis lupus familiaris</i>)		Burkina Faso	
	87BF/2007	Dog (<i>Canis lupus familiaris</i>)		Burkina Faso	
	94BF/2007	Dog (<i>Canis lupus familiaris</i>)		Burkina Faso	
	95BF/2007	Dog (<i>Canis lupus familiaris</i>)		Burkina Faso	
	96BF/2007	Dog (<i>Canis lupus familiaris</i>)		Burkina Faso	
	104BF/2007	Dog (<i>Canis lupus familiaris</i>)		Burkina Faso	
	105BF/2007	Dog (<i>Canis lupus familiaris</i>)		Burkina Faso	
	112BF/2007	Dog (<i>Canis lupus familiaris</i>)		Burkina Faso	
	114BF/2007	Dog (<i>Canis lupus familiaris</i>)		Burkina Faso	
	132BF/2007	Dog (<i>Canis lupus familiaris</i>)		Burkina Faso	
	138BF/2007	Dog (<i>Canis lupus familiaris</i>)		Burkina Faso	
	139BF/2007	Dog (<i>Canis lupus familiaris</i>)		Burkina Faso	
	141BF/2007	Dog (<i>Canis lupus familiaris</i>)		Burkina Faso	
	142BF/2007	Dog (<i>Canis lupus familiaris</i>)		Burkina Faso	
	143BF/2007	Dog (<i>Canis lupus familiaris</i>)		Burkina Faso	
	246NIG/07	Dog (<i>Canis lupus familiaris</i>)		Niger	
	247NIG/07	Dog (<i>Canis lupus familiaris</i>)		Niger	
	251NIG/07	Dog (<i>Canis lupus familiaris</i>)		Niger	
	252NIG/07	Dog (<i>Canis lupus familiaris</i>)		Niger	
	1920MAU/05	Dog (<i>Canis lupus familiaris</i>)		Mauritania	
	2019MAU/06	Dog (<i>Canis lupus familiaris</i>)		Mauritania	
	2049MAU/07	Goat (<i>Capra hircus</i>)		Mauritania	
	1941MAU/06	Dog (<i>Canis lupus familiaris</i>)		Mauritania	
	1923MAU/05	Dog (<i>Canis lupus familiaris</i>)		Mauritania	
	1929MAU/06	Dog (<i>Canis lupus familiaris</i>)		Mauritania	

1922MAU/05	Dog (<i>Canis lupus familiaris</i>)		Mauritania	
RT2006/F	Dog (<i>Canis lupus familiaris</i>)		Tunisia	
RT2009/5	Dog (<i>Canis lupus familiaris</i>)		Tunisia	
86131TUN	Human (<i>Homo sapiens</i>)	Mouse* (<i>Mus musculus</i>)	Tunisia	Yes (positive)
Ariana 2	Dog (<i>Canis lupus familiaris</i>)	Mouse* (<i>Mus musculus</i>)	Tunisia	
8801CAM	Dog (<i>Canis lupus familiaris</i>)	Mouse* (<i>Mus musculus</i>)	Cameroon	
9021CHA	Dog (<i>Canis lupus familiaris</i>)		Chad	
9218CHA	Dog (<i>Canis lupus familiaris</i>)	Mouse* (<i>Mus musculus</i>)	Chad	
9233GAB	Dog (<i>Canis lupus familiaris</i>)		Gabon	Yes (positive)
9239CI	Dog (<i>Canis lupus familiaris</i>)	Mouse* (<i>Mus musculus</i>)	Ivory Coast	
9618ALG	Human (<i>Homo sapiens</i>)		Algeria	Yes (positive)
08170ALG	Cat (<i>Felis catus</i>)		Algeria	Yes (positive)
08177ALG	Dog (<i>Canis lupus familiaris</i>)		Algeria	
08198ALG	Dog (<i>Canis lupus familiaris</i>)		Algeria	Yes (positive)
08056ALG	Dog (<i>Canis lupus familiaris</i>)		Algeria	Yes (positive)
08130ALG	Dog (<i>Canis lupus familiaris</i>)		Algeria	Yes (positive)
08022MAR	Bovine (<i>Bos taurus</i>)		Morocco	Yes (positive)
08030MAR	Cat (<i>Felis catus</i>)		Morocco	Yes (positive)
08050MAR	Dog (<i>Canis lupus familiaris</i>)		Morocco	Yes (positive)
BNVL2009/3580	Dog (<i>Canis lupus familiaris</i>)		Botswana	
BNVL2009/5980	Dog (<i>Canis lupus familiaris</i>)		Botswana	
BNVL2010/627	Bovine (<i>Bos taurus</i>)		Botswana	
BNVL2009/3416	Goat (<i>Capra hircus</i>)		Botswana	
BNVL2009/7331	Goat (<i>Capra hircus</i>)		Botswana	
BNVL2009/5336	WildCat (<i>Felis silvestris silvestris</i>)		Botswana	
BNVL2009/4125	Bovine (<i>Bos taurus</i>)		Botswana	
BNVL2009/2871	Bovine (<i>Bos taurus</i>)		Botswana	
BNVL2009/6665	Honey Badger (<i>Mellivora capensis</i>)		Botswana	
BNVL2009/4112	Bovine (<i>Bos taurus</i>)		Botswana	
BNVL2008/4738	Bovine (<i>Bos taurus</i>)		Botswana	

	BNVL2010/78 8	Bovine (<i>Bos taurus</i>)		Botswana	
	BNVL2010/25 96	Dog (<i>Canis lupus familiaris</i>)		Botswana	
<i>Americas</i>					
	9103USA	Bat (undetermined species)	Mouse* (<i>Mus musculus</i>)	USA	Yes (positive)
	9105USA	Fox (<i>Vulpes vulpes</i>)		USA	
	8909GUY	Bovine (<i>Bos taurus</i>)	Mouse* (<i>Mus musculus</i>)	French Guyana	
	9001GUY	Dog (<i>Canis lupus familiaris</i>)		French Guyana	Yes (positive)
	9704ARG	Bat (<i>Tadarida brasiliensis</i>)	Mouse* (<i>Mus musculus</i>)	Argentina	Yes (positive)
	9705GUY	Bovine (<i>Bos taurus</i>)		French Guyana	Yes (positive)
	03001GUY	Dog (<i>Canis lupus familiaris</i>)		French Guyana	
<i>Asia</i>					
	9902NEP	Goat (<i>Capra hircus</i>)	Mouse* (<i>Mus musculus</i>)	Nepal	Yes (positive)
	9915BIR	Dog (<i>Canis lupus familiaris</i>)	Mouse* (<i>Mus musculus</i>)	Myanmar	Yes (positive)
	94273PHI	Dog (<i>Canis lupus familiaris</i>)	Mouse* (<i>Mus musculus</i>)	Philippines	Yes (positive)
	0116VNM	Dog (<i>Canis lupus familiaris</i>)	Mouse* (<i>Mus musculus</i>)	Vietnam	Yes (positive)
	0117VNM	Dog (<i>Canis lupus familiaris</i>)	Mouse* (<i>Mus musculus</i>)	Vietnam	Yes (positive)
	0240CHI	Human (<i>Homo sapiens</i>)	Mouse* (<i>Mus musculus</i>)	China	Yes (positive)
	0246CHI	Dog (<i>Canis lupus familiaris</i>)	Mouse* (<i>Mus musculus</i>)	China	Yes (positive)
	04035AFG	Dog (<i>Canis lupus familiaris</i>)	Mouse* (<i>Mus musculus</i>)	Afghanistan	Yes (positive)
	8664THA	Dog (<i>Canis lupus familiaris</i>)	Mouse* (<i>Mus musculus</i>)	Thailand	
	117/96	Human (<i>Homo sapiens</i>)		Italy (from Nepal)	
	8677MAL	Human (<i>Homo sapiens</i>)	Mouse* (<i>Mus musculus</i>)	Malaysia	
<i>Fixed strai</i>					
	9503TCH	Vaccinal strain	Mouse* (<i>Mus musculus</i>)	SAD strain, Vnukovo isolate	Yes (positive)
	9507TCH	Vaccinal strain	Mouse* (<i>Mus musculus</i>)	SAD strain, Tubingen isolate	Yes (positive)
	9508TCH	Vaccinal strain	Mouse* (<i>Mus musculus</i>)	SAD strain, Bern-C isolate	Yes (positive)
	9509TCH	Vaccinal strain	Mouse* (<i>Mus musculus</i>)	SAD strain, Postdam	Yes (positive)
	CVS IP13	Fixed strain	Mouse* (<i>Mus musculus</i>)	Challenge virus strain	
	LEP	Vaccinal strain	Chicken embryo fibroblasts		Yes (positive)
	HEP	Vaccinal strain	Chicken embryo		Yes (positive)

			fibroblasts		
	SAG2	Vaccinal strain	Cell supernatant		
	ERA	Fixed strain	Mouse* (<i>Mus musculus</i>)	Evelyn Rokitnicki Abelseth strain	Yes (positive)
	7172-11/10RS	Fixed strain	Mouse** (<i>Mus musculus</i>)	Challenge Virus Strain 11	Yes (positive)
	7172-12/10RS	Fixed strain	Mouse** (<i>Mus musculus</i>)	Challenge Virus Strain 11	Yes (positive)
	7172-13/10RS	Fixed strain	Mouse** (<i>Mus musculus</i>)	Challenge Virus Strain 11	Yes (positive)
	7172-14/10RS	Fixed strain	Mouse** (<i>Mus musculus</i>)	Challenge Virus Strain 11	Yes (positive)
	7172-15/10RS	Fixed strain	Mouse** (<i>Mus musculus</i>)	Challenge Virus Strain 11	Yes (positive)
	7172-16/10RS	Fixed strain	Mouse** (<i>Mus musculus</i>)	Challenge Virus Strain 11	Yes (positive)
	7172-17/10RS	Fixed strain	Mouse** (<i>Mus musculus</i>)	Challenge Virus Strain 11	Yes (positive)
	7172-18/10RS	Fixed strain	Mouse** (<i>Mus musculus</i>)	Challenge Virus Strain 11	Yes (positive)
	7172-19/10RS	Fixed strain	Mouse** (<i>Mus musculus</i>)	Challenge Virus Strain 11	Yes (positive)
	7172-20/10RS	Fixed strain	Mouse** (<i>Mus musculus</i>)	Challenge Virus Strain 11	Yes (positive)
LBV	8619NIG	Bat (<i>Eidolon helvum</i>)	Mouse* (<i>Mus musculus</i>)	Nigeria	Yes (positive)
MOKV	86100CAM	Shrew (<i>Crocidura sp.</i>)	Mouse* (<i>Mus musculus</i>)	Cameroon	Yes (positive)
	86101RCA	Rodent	Mouse* (<i>Mus musculus</i>)	Central African Republic	Yes (positive)
DUVV	86132SA	Human (<i>Homo sapiens</i>)		South Africa	Yes (positive)
	94286SA	Bat (<i>Miniopterus sp.</i>)	Mouse* (<i>Mus musculus</i>)	South Africa	Yes (positive)
EBLV-1	122938	Bat (<i>Eptesicus serotinus</i>)	Mouse* (<i>Mus musculus</i>)	France	
	7102-5/10RS	Bat (<i>Eptesicus serotinus</i>)	Mouse** (<i>Mus musculus</i>)	(8918FRA) France	
	7102-6/10RS	Bat (<i>Eptesicus serotinus</i>)	Mouse** (<i>Mus musculus</i>)	(8918FRA) France	
	7102-7/10RS	Bat (<i>Eptesicus serotinus</i>)	Mouse** (<i>Mus musculus</i>)	(8918FRA) France	
	121411	Bat (<i>Eptesicus serotinus</i>)	Mouse* (<i>Mus musculus</i>)	France	
EBLV-2	9337SWI	Bat (<i>Myotis daubentonii</i>)	Mouse* (<i>Mus musculus</i>)	Switzerland	Yes (positive)
	RT2006/H	Bat (<i>Myotis daubentonii</i>)	Mouse* (<i>Mus musculus</i>)	UK	
ABLV	9810AUS	Bat (<i>Pteropus sp.</i>)	Mouse* (<i>Mus musculus</i>)	Australia	Yes (positive)

Annex 3. Area covered by ORV from 2009 to 2011 and rabies cases diagnosed from 2008 to 2011. The area outlined in pink corresponds to the distribution zone of SADB19 vaccine baits from December 2009 - January 2010, covering approximately 9000 km². During this emergency campaign vaccine baits were distributed only at altitudes below the freezing level (1000m above sea-level), based on average winter temperatures in the affected regions. From 2009 to 2011 a total area of 30,000 km² was covered by ORV campaigns. Rabies cases are indicated as coloured spots according to the period of detection.



Annex 4. List of samples used for the development of the pyrosequencing protocol for differentiating infected from vaccinated animals.

A. Vaccine strains and field samples used in this study for the development of the pyrosequencing protocol. Each sample was previously tested positive by FAT as a standard method whenever applicable, and typed by pyrosequencing with confirmation by Sanger sequencing. Identification matched perfectly with Sanger sequencing results for all tested samples.

* kindly provided by Dr. Laurent Dacheux and Dr. Hervé Bourhy, Institute Pasteur-Paris.

Strain	Host/vector species	Source of sample	Country of origin
Fixed strains			
9503TCH*	Fixed strain	Mouse (<i>Mus musculus</i>)	Street Alabama Dufferin, Vnukovo
9507TCH*	Fixed strain	Mouse (<i>Mus musculus</i>)	Street Alabama Dufferin, Tubingen
9509TCH*	Fixed strain	Mouse (<i>Mus musculus</i>)	Street Alabama Dufferin, Postdam
ERA*	Fixed strain	Mouse (<i>Mus musculus</i>)	Evelyn Rokitnicki Abelseth
SAG2	Fixed strain	Vaccine formulation	Street Alabama Gif
SAD B19	Fixed strain	Vaccine formulation-	Street Alabama Dufferin Bern 19
Africa			
2BF/2007	Dog (<i>Canis lupus familiaris</i>)		Burkina Faso
105NIG/252	Dog (<i>Canis lupus familiaris</i>)		Niger
1920MAU/05	Dog (<i>Canis lupus familiaris</i>)		Mauritania
2019MAU/06	Dog (<i>Canis lupus familiaris</i>)		Mauritania
2029MAU/07	Dog (<i>Canis lupus familiaris</i>)		Mauritania
1978MAU/06	Dog (<i>Canis lupus familiaris</i>)		Mauritania
BNVL2009/6665	Honey Badger (<i>Mellivora capensis</i>)		Botswana
BNVL2009/5336	Wildcat		Botswana
Americas			
9105USA*	Fox (<i>Vulpes vulpes</i>)		USA

9704ARG*	Bat (<i>Tadarida brasiliensis</i>)	Mouse (<i>Mus musculus</i>)	Argentina
9705GUY*	Bovine (<i>Bos taurus</i>)		French Guyana
03001GUY*	Dog (<i>Canis lupus familiaris</i>)		French Guyana
Asia			
9902NEP*	Goat (<i>Capra hircus</i>)	Mouse (<i>Mus musculus</i>)	Nepal
9915BIR*	Dog (<i>Canis lupus familiaris</i>)	Mouse (<i>Mus musculus</i>)	Myanmar
0116VNM*	Dog (<i>Canis lupus familiaris</i>)	Mouse (<i>Mus musculus</i>)	Vietnam
0117VNM*	Dog (<i>Canis lupus familiaris</i>)	Mouse (<i>Mus musculus</i>)	Vietnam
04035AFG*	Dog (<i>Canis lupus familiaris</i>)	Mouse (<i>Mus musculus</i>)	Afghanistan
8677MAL*	Human (<i>Homo sapiens</i>)	Mouse (<i>Mus musculus</i>)	Malaysia

B. List of the Italian samples tested positive for rabies during the recent 2008-2011 epidemic. All the samples were tested as original brain specimens and no further passages in mice or cell cultures were attempted.

Strain	Host	Country of origin
<i>2008</i>		
1981/08RS	Fox (<i>Vulpes vulpes</i>)	Italy
2088/08RS	Fox (<i>Vulpes vulpes</i>)	Italy
2330/08RS	Fox (<i>Vulpes vulpes</i>)	Italy
2396/08RS	Fox (<i>Vulpes vulpes</i>)	Italy
2445/08RS	Fox (<i>Vulpes vulpes</i>)	Italy
2487/08RS	Fox (<i>Vulpes vulpes</i>)	Italy
2624/08RS	Fox (<i>Vulpes vulpes</i>)	Italy
2628/08RS	Fox (<i>Vulpes vulpes</i>)	Italy
2665/08RS	Badger (<i>Meles meles</i>)	Italy
<i>2009</i>		
25/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
134/09RS	Badger (<i>Meles meles</i>)	Italy
209/09RS	Roe deer (<i>Capreolus capreolus</i>)	Italy
556/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
578/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
770/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
899/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
1346/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
1452/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
1459/09RS	Dog (<i>Canis lupus familiaris</i>)	Italy
1964/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
2051/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
2115/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
2116/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
2172/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
2369/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
2417/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
2420/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
2439/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
2472/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
2474/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
2536/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
2616/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
2617/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
2801/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
2846/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
2904/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
2906/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
2973/09RS	Fox (<i>Vulpes vulpes</i>)	Italy

3042/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
3155/09RS	Dog (<i>Canis lupus familiaris</i>)	Italy
3176/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
3234/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
3236/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
3269/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
3270/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
3310/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
3342/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
3424/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
3425/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
3433/09RS	Donkey (<i>Equus asinus</i>)	Italy
3466/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
3473/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
3476/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
3509/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
3510/09RS	Dog (<i>Canis lupus familiaris</i>)	Italy
3561/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
3571/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
3590/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
3592/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
3666/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
3694/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
3704/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
3705/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
3710/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
3766/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
3795/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
3796/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
3801/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
3802/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
3806/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
3811/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
3845/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
3875/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
3958/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
3960/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
3966/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
4027/09RS	Fox (<i>Vulpes vulpes</i>)	Italy
2010		
25/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
29/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
42/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
43/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
44/10RS	Fox (<i>Vulpes vulpes</i>)	Italy

46/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
47/10RS	Badger (<i>Meles meles</i>)	Italy
52/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
65/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
92/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
93/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
94/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
95/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
103/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
104/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
157/10RS	Stone marten (<i>Martes foina</i>)	Italy
209/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
213/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
215/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
223/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
231/10RS	Badger (<i>Meles meles</i>)	Italy
236/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
237/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
309/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
310/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
378/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
380/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
383/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
388/10RS	Roe deer (<i>Capreolus capreolus</i>)	Italy
389/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
394/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
395/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
396/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
400/10RS	Cat (<i>Felis catus</i>)	Italy
401/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
409/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
410/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
414/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
476/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
480/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
484/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
486/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
488/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
542/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
549/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
555/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
556/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
557/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
565/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
688/10RS	Fox (<i>Vulpes vulpes</i>)	Italy

689/10RS	Badger (<i>Meles meles</i>)	Italy
690/10RS	Deer (<i>Cervus elaphus</i>)	Italy
693/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
694/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
695/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
702/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
703/10RS	Cat (<i>Felis catus</i>)	Italy
704/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
705/10RS	Cat (<i>Felis catus</i>)	Italy
712/10RS	Cat (<i>Felis catus</i>)	Italy
715/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
721/10RS	Horse (<i>Equus caballus</i>)	Italy
788/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
843/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
856/10RS	Roe deer (<i>Capreolus capreolus</i>)	Italy
867/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
1015/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
1016/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
1017/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
1018/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
1023/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
1072/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
1073/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
1074/10RS	Badger (<i>Meles meles</i>)	Italy
1075/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
1117/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
1119/10RS	Cat (<i>Felis catus</i>)	Italy
1126/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
1127/10RS	Cat (<i>Felis catus</i>)	Italy
1141/10RS	Badger (<i>Meles meles</i>)	Italy
1142/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
1151/10RS	Cat (<i>Felis catus</i>)	Italy
1251/10RS	Roe deer (<i>Capreolus capreolus</i>)	Italy
1252/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
1331/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
1334/10RS	Stone marten (<i>Martes foina</i>)	Italy
1351/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
1389/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
1390/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
1397/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
1400/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
1402/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
1493/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
1494/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
1496/10RS	Fox (<i>Vulpes vulpes</i>)	Italy

1498/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
1513/10RS	Cat (<i>Felis catus</i>)	Italy
1514/10RS	Cat (<i>Felis catus</i>)	Italy
1585/10RS	Roe deer (<i>Capreolus capreolus</i>)	Italy
1612/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
1617/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
1618/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
1619/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
1684/10RS	Roe deer (<i>Capreolus capreolus</i>)	Italy
1709/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
1719/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
1735/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
1832/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
1833/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
1891/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
1893/10SR	Fox (<i>Vulpes vulpes</i>)	Italy
1915/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
1916/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
1917/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
1918/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
1919/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
1920/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
1934/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
1954/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2008/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2009/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2011/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2012/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2014/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2033/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2043/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2045/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2046/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2048/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2049/10rs	Fox (<i>Vulpes vulpes</i>)	Italy
2051/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2052/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2121/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2137/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2141/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2142/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2151/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2153/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2227/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2260/10RS	Roe deer (<i>Capreolus capreolus</i>)	Italy

2261/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2262/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2263/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2264/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2265/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2266/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2269/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2278/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2339/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2340/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2373/10RS	Roe deer (<i>Capreolus capreolus</i>)	Italy
2432/10RS	Badger (<i>Meles meles</i>)	Italy
2433/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2454/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2469/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2470/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2471/10RS	Badger (<i>Meles meles</i>)	Italy
2490/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2497/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2511/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2519/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2520/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2585/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2602/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2603/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2604/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2643/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2644/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2658/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2659/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2660/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2664/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2711/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2778/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2782/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2788/10RS	Stone marten (<i>Martes foina</i>)	Italy
2803/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2804/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2811/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2852/10RS	Stone marten (<i>Martes foina</i>)	Italy
2892/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
2979/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
3009/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
3065/10RS	Marten (<i>Martes martes</i>)	Italy
3066/10RS	Fox (<i>Vulpes vulpes</i>)	Italy

3067/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
3068/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
3095/10RS	Prairie Dog (<i>Cynomys gunnisoni</i>)	Italy
3098/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
3200/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
3263/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
3311/10RS	Badger (<i>Meles meles</i>)	Italy
3313/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
3410/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
3417/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
3569/10RS	Badger (<i>Meles meles</i>)	Italy
3570/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
3636/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
3690/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
3729/10RS	Badger (<i>Meles meles</i>)	Italy
3888/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
4248/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
4449/10RS	Cat (<i>Felis catus</i>)	Italy
4457/10RS	Bovine (<i>Bos taurus</i>)	Italy
5031/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
5066/10RS	Fox (<i>Vulpes vulpes</i>)	Italy
6507/10RS	Badger (<i>Meles meles</i>)	Italy
6698/10RS	Badger (<i>Meles meles</i>)	Italy
7171/10RS	Badger (<i>Meles meles</i>)	Italy
<i>2011</i>		
819/11RS	Fox (<i>Vulpes vulpes</i>)	Italy

Annex 5. Partial alignment of rabies G gene representative sequences. Complete sequence alignment used for primer designing is available upon request. A: amino acidic substitution R333E (residues 4373-4375) in SAG2 sequences; B and C: two nucleotide polymorphisms in all vaccine strains.

	4380	4		
	A	B	C	
GQ918139 CVS-11	TCCTGGAAATGAGAT			
EF206707.1 RV ERA	A.A.T	C	T	
EF206708.1 RV SAD_Bern (Lysvul	A.A.T	C	T	
EF206709.1 RV SAD_B19 (Fuchsor	A.A.T	C	T	
EF206710.1 RV SAD_Bern origina	A.A.T	C	T	
EF206714.1 RV SAD_Bern origina	A.A.T	C	T	
EF206716.1 RV SAD_VA1 (origina	G.A.T	C	T	
EF206717.1 RV SAD1-3670 var_1	A.A.T	C	T	
EF206718.1 RV SAD1-3670 var_2	A.A.T	C	T	
EU182347.1 RV Mutant_Rb/E3-15-	A.A.T	C	T	
EF206720.1 RV SAD_Bern (Sanafo	A.A.T	C	T	
EU886634.1 RV_2004 from Germa	A.A.T	C	T	
EF206715.1 RV SAD_PS/88 (Rabif	A.A.T	C	T	
EF206719.1 RV_SAG_2	GAA.T	C	T	
SAG2	GAA.T	C	T	
SAG2 SN	GAA.T	C	T	
RV SAD STRAIN-9503TCH	A.A.T	C	T	
RV SAD TUBINGEN-9507TCH	A.A.T	C	T	
RV SAD POSTDAM-9509TCH	A.A.T	C	T	
SAD B19	A.A.T	C	T	
ERA	A.A.T	C	T	
9902NEP Goat Nepal	A.A.			
105NIG/252 Dog Niger		G	A	
1920MAU/05 Dog Mauritania		G	A	
2019MAU/06 Dog Mauritania		G	C	A
2029MAU/07 Dog Mauritania		G	C	A
1978MAU/06 Dog Mauritania		G	C	A
2BF/2007 Dog Burkina Faso		G	A	
BNVL2009/6665 Honey badger Bot	A		A	
BNVL2009/5336 Wildcat Botswana	A		A	
9704ARG Bat Argentina	T	A	T	C
9705GUY Bovine French Guyana	T		T	C
03001GUY Dog French Guyana	T		T	
9915BIR Dog Myanmar	T	A		
0116VNM Dog Vietnam	T	A		
0117VNM Dog Vietnam	T	A		
04035AFG Dog Afghanistan	A			
8677MAL Human Malaysia	C	A		
104/10RS Fox Italy	A			
1452/09RS Fox Italy	A			
AF134341 RV 8653YOU EE wolf	A			
AF134339 RV 9142EST NEE racoon	A		G	
AF134335 RV 9339EST NEE racoon		C	G	
AF134345 RV 86107YOU EE red fo	T			
AF134344 RV 8658YOU EE cattle	T			
AF134326 RV 9223FRA WE red fox	A			
AF134328 RV 9616FRA WE sheep	A			
AF134338 RV 9202ALL CE red fox	A			
AF134337 RV 9212ALL CE red fox	A			
AF134340 RV 9383HON EE red fox	A			
U11748 RV Arctic fox 91RABN073				
U27217 RV PA R89	T	T	GC	
U27216 RV FLA 125	T	T	G	
U03766 RV Arctic A1				
U03765 RV Ontario Type 5				

Annex 6. List of brain specimens collected from dogs analysed in this study.

COUNTRY	VIRUS IDENTIFICATION	YEAR	SPECIES
Algeria	96018ALG	1996	Human
Algeria	96018ALG	1996	Human
Algeria	9137ALG	1991	Dog
Benin	8697BEN	1986	Cat
Bukina Faso	9547HAV	1995	Dog
Bukina Faso	8636HAV	1986	Dog
Bukina Faso	Burkina-19	2007	Dog
Bukina Faso	Burkina-20	2007	Dog
Bukina Faso	Burkina-21	2007	Cat
Bukina Faso	Burkina-70	2007	Dog
Bukina Faso	Burkina-124	2007	Dog
Bukina Faso	Burkina-137	2007	Dog
Bukina Faso	Burkina-144	2007	Dog
Bukina Faso	Burkina-37	2007	Dog
Bukina Faso	28BF	2007	Dog
Bukina Faso	36BF	2007	Dog
Bukina Faso	49BF	2007	Dog
Bukina Faso	139BF	2007	Dog
Burundi	9027BUR	1990	Jackal
Cameroon	8801CAM	1987	Dog
Cameroon	8804CAM	1988	Dog
Cameroon	8805CAM	1988	Dog
Cameroon	CAM1-UN	1988	Dog
CAR	07128RCA	2003	Dog
CAR	07072RCA	2000	Dog
CAR	07149RCA	2004	Dog
CAR	07152RCA	2004	Dog
CAR	07154RCA	2005	Dog
CAR	07158RCA	2006	Dog
CAR	07159RCA	2006	Dog
CAR	07189RCA	2007	Dog
CAR	9228CAF	1992	Dog
Chad	9218TCH	1992	Dog
Chad	8731TCH	1987	Dog
Chad	8732TCH	1987	Dog
Chad	8733TCH	1987	Dog
Chad	9021TCH	1990	Dog
Chad	Chad_44_20A	2005-2006	Dog
Chad	Chad2006_60	2005-2006	Dog
Chad	Chad_41_198	2005-2006	Dog
Chad	Chad2006_45	2005-2006	Dog
Chad	Chad2006_61	2005-2006	Dog

Chad	Chad_42_199	2005-2006	Dog
Chad	Chad2006_43	2005-2006	Dog
Chad	Chad_34_191	2005-2006	Dog
Chad	Chad_33_190	2005-2006	Dog
Chad	Chad_71_164	2005-2006	Dog
Chad	Cahd2006_42	2005-2006	Dog
Chad	Chad_36_193	2005-2006	Dog
Chad	Chad2006_73	2005-2006	Dog
Chad	9609TCH	1996	Dog
DR Congo	8915ZAI	1989	Dog
Egypt	S1	1999	Dog
Egypt	8692EGY	1979	Human
Ethiopia	8808ETH	1988	Dog
Ethiopia	9531ETH	1987	Cow
Ethiopia	8807ETH	1988	Dog
Gabon	8693GAB	1986	Dog
Gabon	8698GAB	1986	Dog
Gambia	080561GMB	2008	Dog
Guinea	8659GUI	1986	Dog
Guinea	8660GUI	1986	Dog
Guinea	9024GUI	1990	Dog
Guinea	9361GUI	1993	Dog
Ivory Coast	GUI1-DG	1986	Dog
Ivory Coast	8911IC	1989	Dog
Ivory Coast	8912IC	1989	Dog
Ivory Coast	8913IC	1989	Dog
Ivory Coast	9003IC	1990	Dog
Ivory Coast	9026IC	1990	Dog
Ivory Coast	9237IC	1992	Dog
Ivory Coast	9238IC	1992	Dog
Ivory Coast	9239IC	1992	Dog
Ivory Coast	07059IC	2007	Dog
Ivory Coast	IVC-UN	1992	Dog
Madagascar	04033MAD	2004	Human
Mali	07211MAL	2007	Dog
Mali	07213MAL	2007	Dog
Mali	07214MAL	2007	Dog
Mali	07217MAL	2007	Dog
Mali	07220MAL	2007	Dog
Mali	07221MAL	2007	Dog
Mali	07222MAL	2007	Dog
Mali	07225MAL	2007	Dog
Mali	07226MAL	2007	Dog
Mali	07227MAL	2006	Dog
Mali	07232MAL	2006	Dog
Mali	07233MAL	2006	Dog

Mali	07235MAL	2006	Dog
Mali	07238MAL	2006	Dog
Mauritania	MAU1-CL	1992	Camel
Mauritania	9120MAU	1991	Dromedary
Mauritania	9131MAU	1991	Dog
Mauritania	9132MAU	1991	Dog
Mauritania	9133MAU	1991	Ass
Mauritania	9136MAU	1991	Goat
Mauritania	9311MAU	1993	Dog
Mauritania	9312MAU	1993	Dog
Mauritania	9314MAU	1993	Dog
Mauritania	8689MAU	1986	Camel
Mauritania	9421MAU	1994	Dog
Mauritania	Mauritania-1920/05	2005	Dog
Mauritania	Mauritania-2019/06	2006	Dog
Mauritania	Mauritania-2049/07	2007	Goat
Mauritania	Mauritania-1941/06	2007	Dog
Mauritania	Mauritania-1923/05	2005	Dog
Mauritania	Mauritania-1929/06	2006	Dog
Mauritania	Mauritania-1922/05	2005	Dog
Morocco	87012MAR	1986	Dog
Morocco	9106MAR	1990	Human
Morocco	8678MAR	1986	Human
Morocco	9016MAR	1990	Dog
Morocco	9108MAR	1991	Human
Morocco	9109MAR	1991	Human
Morocco	9107MAR	1990	Human
Mozambique	8631MOZ	1986	Fox
Namibia	8708NAM	1987	Dog
Namibia	9227NAM	1992	Jackal
Niger	NIG1	1991	Dog
Niger	8718NIG	1987	Dog
Niger	9009NIG	1990	Dog
Niger	9010NIG	1990	Dog
Niger	9011NIG	1990	Dog
Niger	9012NIG	1990	Dog
Niger	9013NIG	1990	Dog
Niger	Niger-246	2007	Dog
Niger	Niger-247	2007	Dog
Niger	Niger-251	2007	Dog
Niger	Niger-252	2007	Dog
Niger	9014NIG	1990	Dog
Nigeria	8670NGA	1986	Human
Nigeria	RD121	2006	Dog
Nigeria	35RD8005	2006	Dog
Nigeria	RD128	2006	Dog

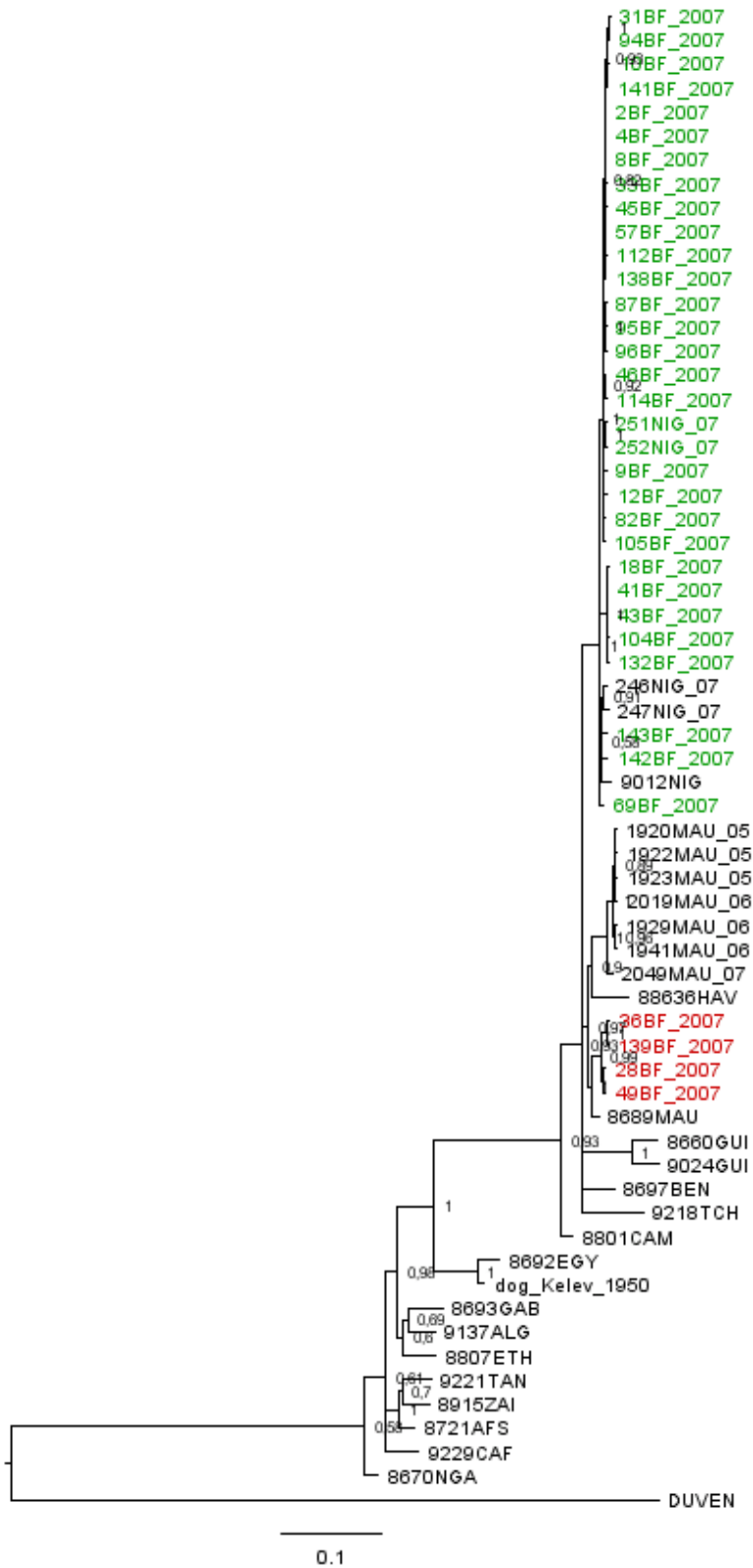
Nigeria	RD141HN	2006	Dog
Nigeria	RD136	2006	Dog
Nigeria	RD42	2006	Dog
Nigeria	RD129	2006	Dog
Nigeria	RD110	2006	Dog
Nigeria	RD111	2006	Dog
Nigeria	RD137	2006	Dog
Nigeria	RD96	2006	Dog
Nigeria	RD100	2006	Dog
Nigeria	RD115	2006	Dog
Nigeria	RD103	2006	Dog
Nigeria	RD41HN	2006	Dog
Nigeria	RD139	2006	Dog
Nigeria	RD9305HN	2006	Dog
Nigeria	RD113HN	2006	Dog
Nigeria	RD119HN	2006	Dog
Rwanda	94289RWD	1995	Dog
Senegal	07209SEN	2007	Human
Senegal	07197SEN	1995	Dog
Senegal	9303SEN	1991	Dog
Senegal	07018SEN	1995	Human
Senegal	07019SEN	1996	Human
Senegal	02025SEN	2001	Human
Senegal	07008SEN	2003	Human
Senegal	07011SEN	2004	Human
Senegal	07021SEN	2004	Human
Senegal	07014SEN	2044	Dog
Senegal	07022SEN	2005	Human
Senegal	07017SEN	2006	Cat
Senegal	07020SEN	2002	Human
Senegal	07009SEN	2003	Human
Senegal	07023SEN	2005	Human
Senegal	07024SEN	2005	Human
Senegal	07027SEN	2006	Human
Senegal	07028SEN	2006	Human
Senegal	07029SEN	2006	Human
Senegal	07207SEN	1997	Human
Sierra Leone	07208SL	1997	Cat
Tanzania	9221TAN	1992	Dog
Tanzania	9222TAN	1992	Cow
Tunisia	8675TUN	1986	Human
Tunisia	8676TUN	1986	Human
Tunisia	86127TUN	1986	Human
Tunisia	86128TUN	1986	Human
Tunisia	86131TUN	1986	Human
Tunisia	8727TUN	1986	Human

Tunisia	86129TUN	1986	Human
Tunisia	86130TUN	1986	Human

Annex 8. RABVs analysed for phylogenetic analyses in Burkina Faso.

GenBank Accession Number	RABV	Origin (town, province, administrative region)	Biting dog
EU478494	2BF/2007	Ouagadougou, Kadiogo, Centre	Y
EU478495	4BF/2007	Tenkodogo, Boulgou, Centre Est	N
EU478496	8BF/2007	Ouagadougou, Kadiogo, Centre	Y
EU478497	9BF/2007	Ouagadougou, Kadiogo, Centre	Y
EU478493	10BF/2007	Ouagadougou, Kadiogo, Centre	Y
EU478498	12BF/2007	Ouagadougou, Kadiogo, Centre	Y
EU478499	18BF/2007	Ouagadougou, Kadiogo, Centre	Y
EU478492	28BF/2007	Kaya, Sanemantenga, Centre-Nord	Y
EU478500	31BF/2007	Ouagadougou, Kadiogo, Centre	Y
EU478501	33BF/2007	Ouagadougou, Kadiogo, Centre	Y
EU478502	36BF/2007	Bobo-Dioulasso, Houet, Hauts Bassins	Y
EU478503	41BF/2007	Ouagadougou, Kadiogo, Centre	Y
EU478505	45BF/2007	Ouagadougou, Kadiogo, Centre	Y
EU478506	46BF/2007	Ouagadougou, Kadiogo, Centre	Y
EU478518	49BF/2007	Bobo-Dioulasso, Houet, Hauts Bassins	Y
EU478507	57BF/2007	Ouagadougou, Kadiogo, Centre	Y
EU478508	69BF/2007	Kaya, Sanemantenga, Centre-Nord	Y
EU478509	82BF/2007	Ziniaré, Ouhimbira, Plateau Central	Y
EU478519	87BF/2007	Ouagadougou, Kadiogo, Centre	Y
EU478520	94BF/2007	Ouagadougou, Kadiogo, Centre	Y
EU478521	95BF/2007	Ouagadougou, Kadiogo, Centre	Y
EU478522	96BF/2007	Ouagadougou, Kadiogo, Centre	Y
EU478510	104BF/2007	Ouagadougou, Kadiogo, Centre	Y
EU478511	105BF/2007	Ouagadougou, Kadiogo, Centre	Y
EU478512	112BF/2007	Ouagadougou, Kadiogo, Centre	Y
EU478513	114BF/2007	Toma, Nayala, Boucle du Mouhoun	Y
EU478523	132BF/2007	Ouagadougou, Kadiogo, Centre	Y
EU478514	138BF/2007	Ouagadougou, Kadiogo, Centre	Y
EU478515	139BF/2007	Bobo-Dioulasso, Houet, Hauts Bassins	N
EU478516	141BF/2007	Ouagadougou, Kadiogo, Centre	Y
EU478524	142BF/2007	Ouagadougou, Kadiogo, Centre	Y
EU478517	143BF/2007	Tenkodogo, Boulgou, Centre-Est	Y

Annex 9. Phylogenetic tree of the nucleoprotein gene constructed by Bayesian methods. Sequences obtained in this study are identified in green (Clade I) and red (Clade II). Posterior probabilities of the clades are indicated above the nodes.



Annex 10. List of 128 fox samples selected as representative of the Italian 2008-2011 epidemic. Sequencing of the complete G gene and intergenic region G-L was performed. The virus identification reveals: the identification number, the species of origin, the month and year of detection, the circumstances of carcass analysis (D: found death; K: killed within the framework of active surveillance), the province of origin.

VIRUS IDENTIFICATION	DAY	MONTH	YEAR	FRAC. YEAR
RS08-1981/fox/oct08/K/Udine	10	10	2008	2008,779
RS08-2088/fox/oct08/D/Udine	19	10	2008	2008,804
RS08-2396/fox/nov08/D/Udine	17	11	2008	2008,882
RS08-2445/fox/nov08/k/Udine	20	11	2008	2008,890
RS08-2624/fox/dic08/K/Udine	17	12	2008	2008,966
RS08-2628/fox/dic08/D/Udine	20	12	2008	2008,974
RS09-25/fox/dic08/D/Udine	30	12	2008	2009,001
RS09-3966/fox/dec09/K/Belluno	24	12	2009	2009,985
RS09-578/fox/mar09/K/Udine	24	3	2009	2009,233
RS09-770/fox/apr09/D/Udine	17	4	2009	2009,297
RS09-1964/fox/jul09/K/Udine	25	7	2009	2009,570
RS09-2801/fox/oct09/K/Pordenone	23	10	2009	2009,815
RS09-3270/fox/nov09/K/Belluno	20	11	2009	2009,890
RS09-3561/fox/dec09/K/Belluno	2	12	2009	2009,925
RS09-3694/fox/dec09/D/Belluno	10	12	2009	2009,947
RS09-3704/fox/dec09/D/Belluno	10	12	2009	2009,947
RS09-3705/fox/dic09/D/Belluno	9	12	2009	2009,944
RS09-3795/fox/dec09/D/Belluno	13	12	2009	2009,955
RS09-3801/fox/dec09/D/Belluno	12	12	2009	2009,952
RS09-3802/fox/dec09/K/Belluno	12	12	2009	2009,952
RS09-3806/fox/dec09/D/Belluno	15	12	2009	2009,960
RS09-3845/fox/dec09/D/Udine	10	12	2009	2009,947
RS09-3958/fox/dec09/K/Belluno	25	12	2009	2009,988
RS10-46/fox/dic09/K/Belluno	30	12	2009	2010,001
RS09-770/fox/apr09/D/Udine	17	4	2009	2009,297
RS09-2616/fox/oct09/D/Pordenone	8	10	2009	2009,774
RS09-2904/fox/oct09/K/Pordenone	31	10	2009	2009,837
RS09-2906/fox/oct09/D/Pordenone	30	10	2009	2009,834
RS09-3042/fox/nov09/D/Pordenone	5	11	2009	2009,849
RS09-3176/fox/nov09/Pordenone	11	10	2009	2009,782
RS09-3310/fox/nov09/D/Udine	22	11	2009	2009,896
RS09-3766/fox/dic09/K/Pordenone	9	12	2009	2009,944
RS09-3710/fox/dic09/D/Belluno	9	12	2009	2009,944
RS09-4027/fox/dic09/D/Belluno	15	12	2009	2009,960
RS09-556/fox/mar09/D/Udine	19	3	2009	2009,219
RS09-899/fox/may09/D/Udine	3	5	2009	2009,342
RS09-1346/fox/jun09/D/Udine	11	6	2009	2009,448
RS09-1452/fox/jun09/D/Udine	22	6	2009	2009,478

RS09-2051/fox/agu09/D/Udine	7	8	2009	2009,604
RS09-2115/fox/agu09/K/Udine	18	8	2009	2009,634
RS09-2116/fox/agu09/D/Udine	18	8	2009	2009,634
RS09-2172/fox/agu09/D/Udine	25	9	2009	2009,737
RS09-2369/fox/sept09/D/Udine	10	9	2009	2009,696
RS09-2420/fox/sept09/D/Udine	18	9	2009	2009,718
RS09-2439/fox/sept09/D/Udine	23	9	2009	2009,732
RS09-2472/fox/sept09/K/Udine	25	9	2009	2009,737
RS09-2474/fox/sept09/k/Udine	26	9	2009	2009,740
RS09-2536/fox/oct09/D/Udine	5	10	2009	2009,766
RS09-2617/fox/oct09/K/Pordenone	6	10	2009	2009,768
RSR09-3234/fox/oct09/D/Belluno	23	10	2009	2009,815
RS09-3236/fox/Nov09/D/Belluno	12	11	2009	2009,868
RS09-3269/fox/nov09/D/Belluno	21	11	2009	2009,893
RS09-3342/fox/nov09/D/Belluno	23	11	2009	2009,899
RS09-3425/fox/nov09/D/Belluno	25	11	2009	2009,904
RS09-3509/fox/nov09/D/Belluno	30	11	2009	2009,918
RS09-3590/fox/dic09/D/Belluno	6	12	2009	2009,936
RS09-3592/fox/dic09/D/Belluno	7	12	2009	2009,938
RS09-3796/fox/dic09/K/Belluno	13	12	2009	2009,955
RS09-3811/fox/dic09/D/Belluno	16	12	2009	2009,963
RS10-213/fox/jan10/D/Belluno	13	1	2010	2010,036
RS10-25/fox/jan10/D/Belluno	2	1	2010	2010,005
RS10-29/fox/jan10/D/Belluno	5	1	2010	2010,014
RS10-44/fox/jan10/K/Belluno	3	1	2010	2010,008
RS10-93/fox/jan10/K/Belluno	6	1	2010	2010,016
RS10-94/fox/jan10/D/Belluno	8	1	2010	2010,022
RS10-104/fox/jan10/D/Belluno	6	1	2010	2010,016
RS10-1709/fox/mar10/D/Udine	9	3	2010	2010,192
RS10-557/fox/jan10/K/Belluno	27	1	2010	2010,074
RS10-215/fox/jan10/K/Belluno	17	1	2010	2010,047
RS10-236/fox/jan10/D/Belluno	18	1	2010	2010,049
RS10-237/fox/jan10/D/Belluno	17	1	2010	2010,047
RS10-380/fox/jan10/D/Belluno	24	1	2010	2010,066
RS10-394/fox/jan10/D/belluno	21	1	2010	2010,058
RS10-396/fox/jan10/D/Belluno	23	1	2010	2010,063
RS10-476/fox/jan10/K/Belluno	25	1	2010	2010,068
RS10-65/fox/jan10/K/Pordenone	5	1	2010	2010,014
RS10-2048/fox/mar10/K/Belluno	29	3	2010	2010,247
RS10-704/fox/feb10/D/Belluno	7	2	2010	2010,103
RS10-1832/fox/mar10/D/Belluno	19	3	2010	2010,219
RS10-1331/fox/mar10/D/Trento	1	3	2010	2010,170
RS10-1351/fox/feb10/D/Udine	28	2	2010	2010,160
RS10-1934/fox/mar10/D/Pordenone	18	3	2010	2010,216
RS10-549/fox/jan10/D/Belluno	28	1	2010	2010,077
RS10-843/fox/feb10/D/Trento	5	2	2010	2010,097

RS10-1126/fox/feb10/D/Udine	9	2	2010	2010,108
RS10-2151/fox/apr10/D/Belluno	3	4	2010	2010,259
RS10-52/fox/jan10/D/Belluno	3	1	2010	2010,008
RS10-1072/fox/feb10/D/Belluno	22	2	2010	2010,144
RS10-1617/fox/mar10/D/Belluno	10	3	2010	2010,195
RS10-1618/fox/mar10/D/Belluno	14	3	2010	2010,205
RS10-2137/fox/apr10/K/Belluno	4	4	2010	2010,262
RS10-1954/fox/mar10/K/Udine	23	3	2010	2010,230
RS10-484/fox/Jan10/K/Belluno	24	1	2010	2010,066
RS10-2121/fox/apr10/K/Pordenone	1	4	2010	2010,253
RS10-2142/fox/apr10/D/Belluno	4	4	2010	2010,262
RS10-2269/fox/apr10/D/Belluno	10	4	2010	2010,278
RS10-2803/fox/apr10/D/Bolzano	19	4	2010	2010,303
RS10-3200/fox/may10/D/Bolzano	23	5	2010	2010,397
RS10-3410/fox/jun10/k/Bolzano	9	6	2010	2010,442
RS10-3636/fox/jun10/D/Trento	12	6	2010	2010,451
RS10-3888/fox/jul10/D/Trento	3	7	2010	2010,510
RS10-5031/fox/agu10/D/Trento	22	8	2010	2010,645
RS10-5066/fox/agu10/K/Belluno	28	8	2010	2010,662
RS10-3570/Fox/Jun10/D/Belluno	21	6	2010	2010,475
RS10-1735/fox/Mar10/Belluno	15	3	2010	2010,208
RS10-2052/fox/mar10/D/Belluno	29	3	2010	2010,247
RS10-2469/fox/apr10/D/Belluno	11	4	2010	2010,281
RS10-2511/fox/apr10/D/Belluno	16	4	2010	2010,295
RS10-2520/fox/apr10/D/Belluno	19	4	2010	2010,303
RS10-2782/fox/May10/K/Belluno	3	5	2010	2010,342
RS10-3098/fox/may10/Belluno	23	5	2010	2010,397
RS10-2892/fox/may10/D/Belluno	10	5	2010	2010,362
RS10-2979/fox/may10/D/Belluno	14	5	2010	2010,373
RS10-3066/fox/may10/D/Belluno	21	5	2010	2010,392
RS10-3263/fox/jun10/D/Belluno	4	6	2010	2010,429
RS10-3313/fox/may10/D/Belluno	8	6	2010	2010,440
RS10-3417/fox/may10/D/Trento	8	6	2010	2010,440
RS10-2643/fox/apr10/D/Belluno	25	4	2010	2010,319
RS10-2711/fox/apr10/D/Udine	24	4	2010	2010,316
RS10-2811/fox/May10/K/Udine	4	5	2010	2010,345
RS10-1719/fox/Mar10/D/Belluno	13	3	2010	2010,203
RS10-1919/fox/Mar10/D/Belluno	21	3	2010	2010,225
RS10-2008/fox/Mar10/D/Belluno	25	3	2010	2010,236
RS10-2009/fox/Mar10/D/Belluno	24	3	2010	2010,233
RS10-2664/fox/Apr10/D/Belluno	26	4	2010	2010,322
RS10-2804/fox/May10/D/Bolzano	1	5	2010	2010,337
RS09-2417/fox/sept09/K/Udine	20	9	2010	2010,723
RS11-819/fox/feb11/D/Belluno	14	2	2011	2011,122

Annex 11. List of primers used for amplification and sequencing of the Italian RABVs. Nucleotide positions are numbered according to the Pasteur virus sequence (GenBank accession number M13215).

Primer name	Length (bp)	Sequence (5'3')	Gene	Position	References
RabForPyro	17	AACACYCTACAATGGA	N	59-75	{{647 De 2011;}}
RabRevPyro-3	22	TCCAATTNGCACACATTTTGTG	N	662-641	{{647 De 2011;}}
RabRevPyro-2	22	TCCARTTAGCGCACATYTTATG	N	662-641	{{647 De 2011;}}
RabRevPyro-2	22	TCCAGTTGGCRCACATCTTRTG	N	662-641	{{647 De 2011;}}
M 220 For ^a	20	TGGTGTATCAACATGRAYTC	M	3000-3019	{{53 Delmas 2008;}}
Rab-Gly-R1 ^b	20	CGRAGCCARTGRTAGTCAGG	G	3742-3723	This study
Rab-Gly-F1 ^b	21	ATGKTTTCCTCWGGYTCTYTTG	G	3318-3338	This study
G780 R ^a	20	ACCCATGTYCCRTCCATAAG	G	4096-4077	{{53 Delmas 2008;}}
Rab-Gly-F3 ^b	21	CAATAGTAGAGGAAGAGAGC	G	3953-3973	This study
Rab-Gly-R3 ^b	20	CCCYTTKGAGGGGATGATYT	G	4403-4384	This study
G780 F ^a	20	CTTATGGAYGGRACATGGGT	G	4077-4096	mod. from {{53 Delmas 2008;}}
Rab-Gly-R4 ^b	20	TCAACMCCTRAGAYYTGTTT	G	4666-4647	This study
Rab-Gly-F5 ^b	20	CCTGAYGGYCAYGTTCTAAT	G	4473-4492	This study
RV-F5	21	GGGGGTGAGACTAGACTGTGA	G	4872-4892	This study
RV-G-REV	21	TGAAAGCACCGTTAGTCACTG	G-L	5119-5099	This study
L1 Rev ^a	21	GAGTTNAGRTTGTARTCAGAG	L	5536-5516	{{53 Delmas 2008;}}