

# EVALUATION OF LONG-TERM PROTECTIVE EFFICACY OF RABIES

## VACCINES IN DOGS

by

XIWEN ZHANG

(Under the Direction of Zhen F. Fu)

### ABSTRACT

Rabies is a fatal encephalitis caused by rabies virus. It causes more than 59,000 human deaths worldwide annually. Rabies vaccination can prevent the development of rabies in humans and animals. In dogs, booster vaccination is administered annually or triennially, as per local law or the vaccines used. To evaluate the long-term efficacy of current rabies vaccines, beagles vaccinated 6 and 8 years ago, were challenged with a virulent rabies virus. Blood and lymph node aspirates were collected for the analysis of immune responses. It was found that 80% of the six-year vaccinated dogs survived with the induction of high levels of virus-neutralizing antibodies (VNA), whereas 80% of the eight-year vaccinated dogs and all of the non-vaccinated dogs succumbed to rabies. Also, six-year vaccinated dogs induced better cellular immune responses than other dogs. These results indicate that a single shot of rabies vaccine can protect dogs for up to six years.

INDEX WORDS: Rabies, rabies vaccines, duration of immunity, immune response, virus-neutralizing antibodies

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IN DOGS

by

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## DEDICATION

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## **CHAPTER 1**

### **INTRODUCTION**

#### **The history of rabies**

Rabies is a very old disease that has been noticed in ancient times. There are many recordings about rabies in myth. Acteon, a famous hunter in one tale, was torn up to death by his own hounds, which are thought to be rabid dogs (George M. Baer, 1991). He used the term “raging dog” or hydrophobia to describe rabies in Ector and Hades (Tsiodras, Korou, Tzani, Tasioudi, Kalachanis, Mangana-Vougiouka, Rigakos, Dougas, Seimenis, & Kontos, 2014). The first description of the disease is recorded around 500 years B.C. by Democritus (George M. Baer, 1991). In the 1st century, Cornelius Celsus, a Greek physician focused on rabies, published that rabies can be transmitted by dog bites (George M. Baer, 1991). He was the first one to use the term rabies (Rupprecht, Hanlon, & Hemachudha, 2002). During the same period, in Roman, Sribonius Largus insisted that there was no chance for a rabid patient to recover (Alan C. Jackson, 2013). At the same time, Eumedes, a physician, observed the obvious sign of hydrophobia, with even several drops of tears enough to excite the convulsion of the pharynx (Fleming, 1872). Then, in about the 2nd century, Magnus of Ephesus started to study the disease in the stomach and diaphragm (Fleming, 1872). About three hundred years later, during the fifth century, Caelius Aurelianus, an ancient authority, concluded from the history and studied the disease carefully and summarized the symptoms of rabies diseases. Preceded by extreme irritability, awkward sitting, wakefulness, nausea, hydrophobia, and fear of sounds, rabies-

infected humans will succumb to death in the end (Fleming, 1872). Even though many physicians and researchers had devoted tremendous efforts to the study of rabies, including pathogenesis, diagnosis, and treatment, there were still many incorrect perspectives of this disease until the early 20<sup>th</sup> century.

With the development of human medical sciences, rabies has become better described in humans. In 1584, Firolamo Fracastoro, an Italian scholar, first recorded the nature of rabies, and the inevitable death following the occurrence of clinical symptoms (Fu, 1997). Until now, there have only been several exceptions (Alvarez, Fajardo, Lopez, Pedroza, Hemachudha, Kamolvarin, Cortes, & Baer, 1994; Centers for Disease & Prevention, 2012; Fuerst, 1966; Hattwick, Weis, Stechschulte, Baer, & Gregg, 1972; Madhusudana, Nagaraj, Uday, Ratnavalli, & Kumar, 2002; Porras, Barboza, Fuenzalida, Adaros, Oviedo, & Furst, 1976; Willoughby, Tieves, Hoffman, Ghanayem, Amlie-Lefond, Schwabe, Chusid, & Rupprecht, 2005). In the hundreds of years since, many scientists and researchers have been devoted to rabies prevention, Zinke, Francois Magendie and Gilbert Breschet showed that rabies can be transmitted by saliva from dogs to rabbits and from dogs to humans (Fu, 1997). With more and more components of rabies elucidated, finally in 1885, based on predecessors' work, Louis Pasteur made the first rabies vaccine (Garg, 2014).

Even though the first rabies vaccine was developed more than one hundred years ago and much progress has been made in this area, rabies remains endemic in most parts of the world. With more than 99% of rabies deaths in humans occurring in the developing countries, rabies has outspread to almost all parts of the world (WHO, 2005a). Nowadays, Western Europe, Canada, the United States of America (USA), Japan, Malaysia, and some

Latin American countries have been regarded as canine rabies-free areas (World Health, 2013). However, rabies still exists in wild animals, such as skunks, raccoons, foxes, raccoon dogs, and bats in these countries. While traveling in those areas, there are chances for humans to get bitten by wild animal virus carriers (World Health, 2013).

### **Rabies epidemiology**

Every year, there are about 59,000 people reported as dying from rabies and more than 7 million have been exposed to rabies (Rupprecht, Willoughby, & Slate, 2006). However, the actual number may be even higher due to underreporting. Most human rabies cases happen in the developing countries of Africa and Asia, where domestic dogs are the major reservoir (Pounder, 2005). Worldwide, about 95% of human rabies is transmitted by dogs (Meslin, 2005), while in the developed countries, bats, foxes, coyotes, raccoons, and skunks are the major reservoirs for rabies (Rupprecht, Willoughby, & Slate, 2006).

In Asia, rabies is a severe public health problem. Approximately 39,000 people die from rabies each year, together with substantial animal welfare, economic, and human health problems (Tenzin & Ward, 2012). The human deaths in Asia account for about 56% of the total human rabies deaths worldwide. India is a typical country with serious rabies problems. It has been reported that rabies exists in almost all regions of the country (Nagarajan, Mohanasubramanian, Seshagiri, Nagendrakumar, Saseendranath, Satyanarayana, Thiagarajan, Rangarajan, & Srinivasan, 2006; Nagarajan, Nagendrakumar, Mohanasubramanian, Rajalakshmi, Hanumantha, Ramya, Thiagarajan, & Srinivasan, 2009). In comparison to other countries, India has the highest rate of human rabies, with an estimated annual 20,000 human deaths from the disease (2.86/100,000 population)

(Sudarshan, Madhusudana, Mahendra, Rao, Narayana, Rahman, Meslin, Lobo, Ravikumar, & Gangaboraiah, 2007). The majority of human rabies in India are caused by dog bites, with the remaining 3% from cats and wildlife (Sudarshan, Mahendra, Madhusudana, Ashwoath Narayana, Rahman, Rao, F, Lobo, Ravikumar, & Gangaboraiah, 2006). However, it is unfortunate that the Indian government has not regarded rabies as an important infectious disease, despite the high rabies mortality (Sudarshan, Madhusudana, Mahendra, Rao, Ashwath Narayana, Abdul Rahman, Meslin, Lobo, Ravikumar, & Gangaboraiah, 2007). In Pakistan, rabies is also endemic. It causes around 4,000 human deaths every year (Jamali, 2001). Pet animals in Pakistan are mostly vaccinated under the care of veterinarians. However, there are lots of stray dogs that remain unvaccinated. In China, rabies cases are also reported in almost all provinces (Hu, Tang, Tang, & Fooks, 2009). Most human rabies cases are reported in rural areas (Hu, Tang, Tang, & Fooks, 2009; Song, Tang, Wang, Mo, Guo, Li, Tao, Rupprecht, Feng, & Liang, 2009). Rural street dogs are responsible for more than 95% of the human rabies cases. The two major reasons that rabies is endemic in developing countries are the substantial economic burden associated with rabies control and the lacking understanding of the true public health impact of rabies (Cleaveland, Kaare, Knobel, & Laurenson, 2006). In addition, other reasons, include poor control of the stray dogs in rural areas, and the high price of vaccines for animals. Well-organized rabies control programs, particularly rabies vaccination, are extremely needed in these developing countries.

In the developed countries, such as the US, wild animals account for more than 90% of reported rabid animal cases. In Western and Southern Europe, canine rabies gradually disappeared from most countries during the first half of the 20<sup>th</sup> century, and

foxes contributed to 83% rabid animals before the use of wildlife vaccination (Finnegan, Brookes, Johnson, Smith, Mansfield, Keene, McElhinney, & Fooks, 2002). In 2011, wild animals resulted in 91.8% of the 6,031 rabid animals reported in the USA, while domestic animals only contributed 8.2% of rabies cases. Rabies has been well controlled for more than 55 years. For the last three decades, most human rabies cases have been associated with rabies viruses carried by wildlife, particularly bats (Anderson, Nicholson, Tauxe, & Winkler, 1984; Messenger, Smith, & Rupprecht, 2002). However, there are also some other important animal reservoirs contributing to rabies maintenance, such as raccoons, foxes, skunks, and mongooses. During the past three decades after intensive implementation of oral rabies vaccination programs, fox-mediated rabies from vast areas of Western and Central Europe have been eliminated (Freuling, Hampson, Selhorst, Schroder, Meslin, Mettenleiter, & Muller, 2013).

### **Rabies virus**

Rabies virus (RABV) is an enveloped, bullet-shaped, negative-stranded RNA virus, belonging to the genus *Lyssavirus*, family *Rhabdoviridae*. The genome of rabies virus is about 12kb in length, encoding for five proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and polymerase (L).

The family *Rhabdoviridae* consists of 6 genera and more than 100 viruses with a wide range of hosts, in both plants and animals, including mammals, birds, reptiles, and fish (Kumin, Novella, Dietzgen, Padhi, & Rupprecht, 2009). A bullet- or rod-shaped morphology is the common shape of the viruses in this family (Kumin, Novella, Dietzgen, Padhi, & Rupprecht, 2009). *Cytorhabdovirus* causes diseases in plants,

*Novirhabdovirus* can cause diseases in fish and other aquatic animals, and *Vesiculovirus* and *Ephemerovirus* can result in diseases in livestock (Kumin, Novella, Dietzgen, Padhi, & Rupprecht, 2009). Infection with these viruses can have a great impact on the economies (Kuzmin, Hughes, & Rupprecht, 2006). However, *Lyssavirus*, the most important genus in the family of *Rhabdoviridae*, includes the deadly rabies virus, which causes human deaths and huge economic losses.

There are fourteen *Lyssaviruses*, divided into two large phylogroups according to genetic diversity, pathogenicity, and immunogenicity (Alan C. Jackson, 2013). The two phylogroups are divided into seven genotypes. Phylogroup I includes the classic (prototype) rabies virus (RABV, genotype 1), Duvenhage virus (DUVV, genotype 4), the European bat lyssavirus types 1 and 2 (EBL1, EBL2, genotype 5), and the Australian bat lyssavirus (ABLV, genotype 7). Phylogroup II comprises Lagos bat virus (LBV, genotype 2) and Mokola virus (MOKV, genotype 3) (Badrane, Bahloul, Perrin, & Tordo, 2001).

The viruses in the family of *Rhabdoviridae*, which means rod in Greek, all have similar shapes. They are all rod- or bullet-shaped with lengths varying from 130 to 380nm and widths varying from 60 to 95nm (Brown, Bishop, Crick, Francki, Holland, Hull, Johnson, Martelli, Murphy, Obijeski, Peters, Pringle, Reichmann, Schneider, Shope, Simpson, Summers, & Wagner, 1979). The rabies virus particle is about 180 by 75 nm, having a knoblike structure at one end and a smooth, flat opposite end (Hummeler, Koprowski, & Wiktor, 1967). There are two membranes, with many protrusions on the surface, of different densities surrounding the virus and one inner filamentous material core (Hummeler, Koprowski, & Wiktor, 1967). Inside the inner core, ribonucleoprotein (RNP),



is comprised of three proteins, N, P, and L. The outer membrane consists of membrane proteins, G and M (Alan C. Jackson, 2013).

### **N protein**

The rabies virus N protein consists of 450 amino acids (Alan C. Jackson, 2013). It is the most conserved protein in all of the lyssaviruses. However, it also has a relatively high degree of genetic diversity between genotypes (Marston, McElhinney, Johnson, Muller, Conzelmann, Tordo, & Fooks, 2007).

N protein is a phosphorylated protein and plays an important role in virus transcription and replication. It is phosphorylated by cellular casein kinase II (X. Wu, Lei, & Fu, 2003). It regulates transcription and replication of the virus by modulation of leader RNA encapsulation (J. Yang, Koprowski, Dietzschold, & Fu, 1999). If the nucleoprotein is not phosphorylated, the level of both viral transcription and replication will be reduced, and viral production will decrease by as much as 10,000-fold (X. F. Wu, Gong, Foley, Schnell, & Fu, 2002).

N is an important antigenic protein in lyssavirus, and it is very crucial for the immune responses. There are several linear epitopes and conformational epitopes on the N protein. The linear epitopes are suitable for rabies virus diagnosis, while the conformational epitopes can be used for probing the structure of N protein (Jiang, Luo, Michel, Hogan, He, & Fu, 2010). Capable of inducing helper T cells, the N protein is a major antigen of rabies virus. There are three antigenic sites on nucleoprotein (Lafon & Wiktor, 1985). There are also some epitopes of the rabies virus nucleoprotein that induce B cells and T cells (da Cruz, McBride, Conceicao, Dale, McFadden, & Dellagostin, 2001). Since the rabies virus N is highly antigenic to activate B cells to produce antibodies and T helper

cells, it was used as a vaccine component against rabies virus and also as a vector for other diseases (Koser, McGettigan, Tan, Smith, Koprowski, Dietzschold, & Schnell, 2004).

When expressed in insect cells, purified N protein is still antigenically and immunogenically comparable to the rabies virus ribonucleoprotein (Fu, Dietzschold, Schumacher, Wunner, Ertl, & Koprowski, 1991). Rabies virus N helps to evade activation of the RIG-I-mediated antiviral responses of the host (Masatani, Ito, Shimizu, Ito, Nakagawa, Sawaki, Koyama, & Sugiyama, 2010). In this function, amino acids at positions 273 and 394 play a very important role, which makes the evasion more efficient (Masatani, Ito, Shimizu, Ito, Nakagawa, Abe, Yamaoka, & Sugiyama, 2011).

### **P protein**

The rabies virus P protein consists of 297 or 303 amino acids, accounting for 6% of the total amount of proteins in the virions and it is the most non-conserved protein (Alan C. Jackson, 2013). This monomer consists of two  $\alpha$ -helices, which make a helical, hydrophobic, interacting hairpin (Ivanov, Crepin, Jamin, & Ruigrok, 2010). Like the counterpart in vesicular stomatitis virus, the P protein in rabies virus can form dimers. As an essential cofactor of the virus RNA-dependent RNA polymerase, the P protein plays an important role in virus transcription and replication (Chenik, Chebli, Gaudin, & Blondel, 1994; Li, Dong, Shi, Deng, Chen, Wan, Zhou, Zhao, Fu, & Peng, 2016). P protein interacts with other proteins for more efficient transcription (Tan, Preuss, Williams, & Schnell, 2007). The P protein also interacts with STAT1 and interferon, inhibiting interferon signaling pathways (Vidy, Chelbi-Alix, & Blondel, 2005; Vidy, El Bougrini, Chelbi-Alix, & Blondel, 2007). The P protein interferes with the phosphorylation of interferon regulatory factor 3 and inhibits its nuclear accumulation to prevent interferon responses

(Brzozka, Finke, & Conzelmann, 2005; Rieder, Brzozka, Pfaller, Cox, Stitz, & Conzelmann, 2011; Vidy, El Bougrini, Chelbi-Alix, & Blondel, 2007). In the infected cells, rabies virus P protein can interact with mitochondrial proteins, generating more reactive oxygen species (Kammouni, Wood, Saleh, Appolinario, Fernyhough, & Jackson, 2015).

### **G protein**

Rabies virus G protein consists of 524 amino acids and accounts for 42 percent of the whole virus proteins. The oligomerization state of G is a trimeric structure with a “head” and a “stalk” (Gaudin, Ruigrok, Tuffereau, Knossow, & Flamand, 1992). Low pH can result in conformational change of the G protein, which plays an important role in membrane fusion (Y. Gaudin, R. W. Ruigrok, M. Knossow, & A. Flamand, 1993). Rabies virus G is involved in interacting with receptors, contributing to its neuroinvasiveness and pathogenicity (Faber, Faber, Papaneri, Bette, Weihe, Dietzschold, & Schnell, 2005a). There are many antigenic sites on the G protein, which induces the generation of virus neutralizing antibodies (VNA), providing protection to the host (Cox, Dietzschold, & Schneider, 1977). There are two antigenic sites, sites IIa, and III, which are dominant for the neutralizing antibodies to bind (Benmansour, Leblois, Coulon, Tuffereau, Gaudin, Flamand, & Lafay, 1991; Prehaud, Coulon, LaFay, Thiers, & Flamand, 1988).

### **M protein**

The matrix (M) protein forms the outside layer of the RNP coil, maintaining it in the condensed form (Mebatsion, Weiland, & Conzelmann, 1999a). Acting as a replication-stimulatory factor, the M protein helps to keep the balance between viral replication and transcription (Finke, Mueller-Waldeck, & Conzelmann, 2003). The M protein also plays an important role in virus assembly and budding by getting involved in the cellular

translation machinery and interacting with cellular proteins (Komarova, Real, Borman, Brocard, England, Tordo, Hershey, Kean, & Jacob, 2007; Mebatsion, Weiland, & Conzelmann, 1999a). A proline-rich motif, PPEY, within the M protein, interacts with WW domains of cellular proteins and promotes virus budding (Harty, Paragas, Sudol, & Palese, 1999; Wirblich, Tan, Papaneri, Godlewski, Orenstein, Harty, & Schnell, 2008). The M protein is also associated with cytopathogenicity. The amino acid at position 95 determines the cytopathogenicity of the virus (Mita, Shimizu, Ito, Yamada, Ito, Sugiyama, & Minamoto, 2008).

### **L protein**

In rabies virus, the L and P proteins form the RNA polymerase complex, which plays a crucial role in viral replication and transcription. The dynein light chain 1 (DLC1) binding motif in the L can mediate microtubule (MT) binding through dynein motors, making the L protein accumulate at the acetylated and reorganized MTs (Bauer, Nolden, Nemitz, Perlson, & Finke, 2015). It has been shown that the function of the L protein is related to the mRNA capping, methyltransferase, and poly A polymerase activities (Poch, Blumberg, Bougueleret, & Tordo, 1990).

### **Rabies virus life cycle**

Like most viruses, the life cycle of rabies virus can be divided into three phases. The first phase consists of virus binding and entering into permissive cells by endocytosis, releasing the viral genome into the cytosol after fusion of the viral and endosomal membranes. The second phase is the process of producing virion components, including transcription, replication, and protein synthesis. The third phase is viral component

assembly and exocytosis of the virions. The mature virion will find a new host cell and start a new infection and life cycle.

The very first step of the rabies virus life cycle is virus attachment to cells. Rabies virus G protein binds to cells by interacting with cell surface receptors. There are several receptors that can bind to rabies virus G. Nicotinic acetylcholine receptor is one of the receptors that bind to rabies virus, and the  $\alpha$ -subunit peptide of the receptor is the binding site for the rabies virus (Lentz, 1990). Other than the nicotinic acetylcholine receptors, the neuronal cell adhesion molecule (NCAM), CD56, on the cell surface has also been proposed as a rabies virus receptor (Thoulouze, Lafage, Schachner, Hartmann, Cremer, & Lafon, 1998). NCAM-deficient mice showed decreased mortality and fewer brain lesions (Thoulouze, Lafage, Schachner, Hartmann, Cremer, & Lafon, 1998). The third rabies virus receptors is the low-affinity nerve-growth factor receptor, P75NTR (Tuffereau, Benejean, Blondel, Kieffer, & Flamand, 1998). The binding site in this neurotrophin receptor is cysteine-rich domain 1 (Tuffereau, Schmidt, Langevin, Lafay, Dechant, & Koltzenburg, 2007). Further studies showed that P75NTR is essential for rabies virus infection in primary neurons (Tuffereau, Schmidt, Langevin, Lafay, Dechant, & Koltzenburg, 2007). In addition, sialylated gangliosides, as components of receptor structure, facilitate the binding of rabies virus to infected cells (Superti, Hauttecoeur, Morelec, Goldoni, Bizzini, & Tsiang, 1986).

After binding to the host cell, the virus enters the cells by the endocytic pathway (Le Blanc, Luyet, Pons, Ferguson, Emans, Petiot, Mayran, Demareux, Faure, Sadoul, Parton, & Gruenberg, 2005). Outside of the host cells, the rabies virus particle can be detected both in clathrin-coated pits and in uncoated vesicles (Superti, Derer, & Tsiang,

1984). The G protein will then go through three conformational changes to allow the viral genome to enter the host cell cytoplasm. Then, the rabies virus is transported from the endosome into the cytosol. The pH changes in the endosome will induce changes in the G protein structure and release of the viral capsid. When the pH lowers to 6.4, there will be viral aggregation at the binding site and the G becomes sensitive to proteases. When the pH value decreases to 6.1, the endosomal membrane starts to fuse with viral G (Y. Gaudin, R. W. H. Ruigrok, M. Knossow, & A. Flamand, 1993; Gaudin, Tuffereau, Segretain, Knossow, & Flamand, 1991). Unlike other fusogenic viruses, this conformational change is reversible according to the equilibrium of the pH (Gaudin, Tuffereau, Segretain, Knossow, & Flamand, 1991). So both in the binding and membrane fusion processes, the G protein plays an important role.

The second phase of the viral cycle is the transcription and replication of the viral genome. Once the RNP is released into the cytosol, the viral RNA switches from the condensed and helical pattern to a relaxed form, which is synthetically active (Schnell, McGettigan, Wirblich, & Papaneri, 2010). The negative-sense RNA, which is released in the cytoplasm, is used to produce the template for transcription, which is catalyzed by the RNA polymerase, the L-P polymerase complex (Albertini, Ruigrok, & Blondel, 2011). However, the mechanism of the polymerase complex gaining access to the vRNA is not well-understood. The transcription process starts at the 3'-end of the genomic RNA. The polymerase complex can recognize a specific promotor at the 3'-end and slide to the 5'-end by a mechanism called stop-start, in which transcription is terminated when the polymerase complex encounters a conserved signal sequence (Albertini, Ruigrok, & Blondel, 2011; Schnell, McGettigan, Wirblich, & Papaneri, 2010). Then, six consecutive transcripts are

produced, a short uncapped and unpolyadenylated, leader RNA and five, successive, 5' end-capped and polyadenylated mRNAs coding for the N, P, M, G, and L proteins (Albertini, Ruigrok, & Blondel, 2011; Schnell, McGettigan, Wirblich, & Papaneri, 2010). During transcription, the polymerase complex identifies difference signals, such as initiation, termination, and polyadenylation, which flank the cistrons (Albertini, Ruigrok, & Blondel, 2011). When the complex recognizes the stop signal, it then dissociates from the template and is ready to restart poorly at the next start signal (Albertini, Ruigrok, & Blondel, 2011). The complex continues to transcribe the genome until it reaches the end of the L gene. There is a concentration gradient of the quantity of each mRNA according to their order in the genome,  $N > P > M > G > L$  (Albertini, Ruigrok, & Blondel, 2011).

When enough N proteins are produced, the replication is then activated (Liu, Yang, Wu, & Fu, 2004). The replication process requires two parts that interact with each other. First, the ongoing protein synthesis is needed to produce enough soluble N protein, encapsulating the nascent RNA; second, the polymerase complex switches to replication, producing a complementary, full-length, positive-strand, genomic RNA (Albertini, Ruigrok, & Blondel, 2011). In order to make the N protein bind cellular RNA and not aggregate, the positive-stranded RNAs are encapsulated by N protein and bond to the L-P complex (Peluso & Moyer, 1988). The decorated positive-stranded RNAs are then used as a template to produce the negative-stranded genomic RNA for newly-produced virions (Albertini, Ruigrok, & Blondel, 2011; Schnell, McGettigan, Wirblich, & Papaneri, 2010).

After transcription and replication, the viral life cycle enters into the third phase, virus assembly and release. In this phase, the M protein plays an important role in all negative-stranded RNA viruses, even though the structures of the M proteins in different

viruses have only minor similarities (Schmitt & Lamb, 2004). The M protein is beneath the lipid envelope, which has the benefit of connecting the inner component RNP cores and the outer layer envelope G protein. This process starts when the vRNA is encapsidated and RNP is formed, which needs accumulation of viral proteins N, P, and L (Alan C. Jackson, 2013). The M protein maintains the balance between viral transcription and replication by associating with transcriptionally active RNP (Finke & Conzelmann, 2003). After the formation of the RNP, the M protein makes contact with the RNP complex and helps with viral assembly and exocytosis. After glycosylation, the trimeric G protein is aggregated at the cellular membrane, where the RNP coil is also located (Mebatsion, Weiland, & Conzelmann, 1999b). M protein recognizes the place by localizing the RNP coil and interacts with the G protein (Mebatsion, Weiland, & Conzelmann, 1999b). Then, the RNP is condensed by the M protein into a 'skeleton-like' form, tightly coiled, suspend the polymerase activity (Alan C. Jackson, 2013).

The M protein, probably in the state of a dimer, gets enveloped by the cellular membrane, which is the start of the budding process. The budding process can also be regarded as the final stage of the assembly process. The lipid bilayer envelope is acquired when the assembled components, RNP, and M, bud through the cellular membrane. But this is not the only way for the virion to obtain the lipid bilayer membrane. Some virions mature intracellularly and obtain their outer layer membrane from the Golgi apparatus or cytoplasmic ER (Finke, Granzow, Hurst, Pollin, & Mettenleiter, 2010). When the virion buds from the host cells, it needs to have the G protein anchored in the membrane and in the form of trimeric spike-like structures, which are helpful for efficient budding (Whitt,



Buonocore, Prehaud, & Rose, 1991). The stability of the G protein trimer is enforced by G-M interaction.

### **Rabies pathogenesis and pathology**

Rabies virus is a deadly virus that causes severe neurotropic diseases. The virus travels from the peripheral nerves to the central nervous system (CNS), causing an acute infection. From the entry site of the virus to the CNS, there are several steps, including viral replication in peripheral tissues, traveling through peripheral nerves and the spinal cord to the brain, viral spread within the CNS and back to peripheral organs, especially the salivary glands (Alan C. Jackson, 2013). Changes in cell morphology and damage can be observed in different sites, as the rabies virus spreads through the nervous system. For most cases of rabies virus infection, the viruses are introduced into the body by the bites of rabid dogs, even though there are very few cases showing that the virus can be spread via the airborne route (Winkler, 1968).

### **Clinical signs**

The incubation period of rabies ranges from weeks to months, and even to several years in rare occasions (George M. Baer, 1991; Charlton, Casey, & Campbell, 1987; Hanna, Carney, Smith, Tannenberg, Deverill, Botha, Serafin, Harrower, Fitzpatrick, & Searle, 2000). Many factors contribute to the long and variable incubation period of rabies, including the virus strain, the site of exposure, and the dose. Depending on the strain and dose used, experimentally-infected dogs have incubation periods from 7 to 125 days (Fekadu, 1988). Interestingly, the length of the incubation period has negative correlation with the virus dose (Fekadu, 1988). Since the incubation period is mainly dose dependent,

it means that the incubation periods can be manually decreased by injecting a large amount of the virus.

The classical development of canine rabies can be divided into three phases: prodromal, excitative, and paralytic. There are two types of clinical rabies, furious rabies and dumb rabies. In the first form, the excitative phase is predominant while in the second, the disease progresses from the prodromal to the paralytic phase, because the excitative phase is too short or absent (Fekadu, 1993).

The prodromal phase usually lasts for 1 to 3 days. There may be some behavioral changes. Active and high-strung dogs may become more easy going than usual, while ordinarily friendly dogs may become shy and hide from humans or become irritable and snappy. Some other signs include slightly increased temperature, dilation of the pupils and drowsy eye contact. Importantly, profound salivation may occur.

The excitative phase may last for 1 to 7 days, and the signs of this phase are most easily recognizable. At the early point of this phase, the dog may shun people and hide in the dark. Then, the dog become restless and agitated. It may like to move and roam a lot. This is the most dangerous phase, because the dog becomes violent and will bite anything it encounters, whether human, animal, or inanimate object. In most cases, there is a typical change in the bark, an altered phonation, caused by paralysis of laryngeal muscles. In addition, spasms or even paralysis of the pharyngeal muscles makes it difficult to swallow and leads to drooling. Sometimes, frothing of the drooling salivation occurs due to heavy and rapid respiration. After survival from the characteristic convulsive seizures, the dog enters into the paralytic phase.

The last phase is the paralytic stage. Sometimes, the excitative phase is extremely short or absent, and sick dogs progress directly to the paralytic phase (dumb rabies). In this phase, the disease usually causes muscular incoordination, paralysis, coma, and death. The most characteristic sign is the so-called “dropped jaw”, resulting from paralysis of the masseter muscles, making it impossible to eat or drink. Usually, the dog will make choking sounds, as if bones were stuck in the throat, leading people to attempt to remove the “bones”. This often results in owners scratching their hands on the dog’s teeth and expose to the virus.

### **The immune responses to infection**

During infection, the rabies virus faces host defenses in different ways. Firstly, when rabies virus is delivered to the skin or muscle, it is recognized rapidly by the early line of defense, the innate immune response. This results in not only local elimination of microbes but also the induction of specific immune responses, by B and T cells, in the periphery. After entry into the nerves, the rabies virus needs to counter the innate immune responses induced by the infection. As soon as the infection reaches the neurons, they are protected from elimination by infiltrating T cells and by limiting the inflammation of neuronal tissue. In addition, immunological homeostasis might also facilitate the propagation of the virus in the nervous system (Alan C. Jackson, 2013). Preservation of the integrity of the neuronal network up to the brain stem gives the opportunity for the virus to reach the salivary glands and be transmitted (Alan C. Jackson, 2013).

The innate immune response is the frontline of defense against infectious agents. In the spot where rabies virus is inoculated by bites or scratches, the innate immune

response is triggered in the first few hours after entry of the viruses. This response is not pathogen specific. In the infected neurons, the antiviral, chemoattractive, and inflammatory responses play critical roles. Many components contribute to the innate immune response, including inflammatory cytokines,  $\text{IFN}\alpha/\beta$ ,  $\text{TNF-}\alpha$ ,  $\text{IL-1}$ , and  $\text{IL-6}$ , and the chemokines IP-10,  $\text{MIP-1}\alpha$ , and RANTES, which can be detected in experimentally-infected animals and help to fight against the viruses (Akira, Yamamoto, & Takeda, 2003; A. C. Jackson, Rossiter, & Lafon, 2006; Johnson, McKimmie, Mansfield, Wakeley, Brookes, & Fazakerley, 2006; Wang, Sarmiento, Wang, Li, Dhingra, Tsegai, Jiang, & Fu, 2005).

The activation of the adaptive immune response occurs in the lymphoid organs. The  $\text{CD4}^+$  T cells recognize rabies virus that has been processed by the MHC II pathway by activated dendritic cells (DC). With the help of  $\text{CD4}^+$  T cells, B cells are activated, which is an important part of the adaptive immune response (Garenne & Lafon, 1998). After activation,  $\text{CD4}^+$  T cells can produce cytokines, such as  $\text{IL-4}$ , to trigger VNA production. However, it is not clear how VNA mediate virus clearance. There are two main hypotheses regarding how VNA act on rabies virus. One hypothesis is called “steric hindrance”. In this theory, VNA can restrain virus attachment to host cells by blocking all G protein spikes on the surface of the virions (Irie & Kawai, 2002). Another hypothesis states that the virion’s ability to attach to host cells is restrained, but in a different way, through conformational changes of the G protein spikes induced by VNA (Irie & Kawai, 2002). In terms of  $\text{CD8}^+$  T cells, they show no measurable effect on protecting the host against RABV (Perry & Lodmell, 1991).

## **Rabies vaccines**

Rabies virus causes lethal disease in human and animals, therefore prompt vaccination is very useful in preventing the disease prior to or post exposure. There are several vaccines that are commercially available for animals. Inactivated vaccines are most widely used in domestic animals, while live-attenuated vaccines or live recombinant vaccines have been developed and produced as oral vaccines for wild animals.

### **Live recombinant canarypox vaccine expressing RABV G protein**

In addition to the inactivated vaccines, there is a live recombinant canarypox vaccine expressing rabies G protein for cats (Cadoz, Strady, Meignier, Taylor, Tartaglia, Paoletti, & Plotkin, 1992). It can not only be used as a monovalent vaccine for cats, but it can also be used as a multivalent vaccine that includes rabies, feline panleukopenia virus, calicivirus, parvovirus, and chlamydia psittaci (Poulet, Minke, Pardo, Juillard, Nordgren, & Audonnet, 2007). The host-restricted vector of the vaccine, canarypox virus vector or ALVAC, can only infect and cause diseases in avian species. Even though there is interrupted replication in non-avian cells, the canarypox virus vector is able to express foreign antigens during the early phase of vector replication and effectively stimulates immune responses. Many animal species have been tested in clinical trials with this vaccine, showing a high level of safety and efficacy (Tartaglia, Jarrett, Neil, Desmettre, & Paoletti, 1993). The use of adjuvant in inactivated vaccines to induce a strong immune response is related with feline fibrosarcomas. However, the live recombinant canarypox vaccine can stimulate protective immune responses without the help of adjuvants, which is a huge advantage (Barber, Sorenmo, Cronin, & Shofer, 2000).

## **Oral rabies vaccines for wild animals**

Since the 1970s, rabies in dogs has been well controlled in the US. However, rabies in wild animals continues to present a public health threat. More than 90% of rabies cases are reported in wild animals, which was a real threat to humans and domestic animals (Anderson, Nicholson, Tauxe, & Winkler, 1984; Messenger, Smith, & Rupprecht, 2002). The first oral rabies vaccine licensed in the USA is RABORAL® V-RG. This oral rabies vaccine was developed in 1984 when a recombinant vaccinia virus (V-RG) expressing rabies virus G protein was successfully constructed (Kieny, Lathe, Drillien, Spehner, Skory, Schmitt, Wiktor, Koprowski, & Lecocq, 1984). This recombinant vaccinia virus was constructed from the Copenhagen strain of vaccinia virus, and the thymidine kinase gene was deleted, and replaced by the cDNA of the rabies virus G protein (Kieny, Lathe, Drillien, Spehner, Skory, Schmitt, Wiktor, Koprowski, & Lecocq, 1984). Due to the advantage of oral administration, the V-RG vaccines are mostly distributed by aircraft, helicopter, or even by drones, in different seasons in the wild (Selhorst, Muller, & Batza, 2006). However, in some urban and suburban areas, manual distribution of vaccine baits is also preferably used as a complementary measure (Masson, Bruyere-Masson, Vuillaume, Lemoyne, & Aubert, 1999). Distribution in the wild requires the V-RG vaccine to have great stability. The vaccine stays stable above 56°C, while the bait-casing melting point can be as high as 60°C (Brochier, Blancou, Thomas, Languet, Artois, Kieny, Lecocq, Costy, Desmettre, Chappuis, & Pastoret, 1989). The vaccine has also been tested in over 10 avian and 35 mammalian species, including the majority of rabies reservoir hosts, with the results showing that it is safe for all of these animals. (Brochier, Blancou, Thomas, Languet, Artois, Kieny, Lecocq, Costy, Desmettre, Chappuis, & Pastoret, 1989). V-RG

vaccine has been used to control rabies in the coyote population along the US-Mexico border, which was a great success in eliminating coyote rabies from the USA in the 1990s (Fearneyhough, Wilson, Clark, Smith, Johnston, Hicks, & Moore, 1998; Sidwa, Wilson, Moore, Oertli, Hicks, Rohde, & Johnston, 2005).

The second one is ONRAB®, licensed in Canada. This oral vaccine for wild animals is based on human adenovirus type 5 (HAd5). The live recombinant virus was constructed by replacing the E3 region of the adenovirus genome with rabies G protein, in order to decrease the expression of the major histocompatibility complex (MHC) I antigens and free the virus-infected cells from T cells attack (Wold & Gooding, 1991; Y. P. Yang, Ertl, & Wilson, 1994). Genetically and phenotypically, the vaccine has been proven to be stable for up to 10 passages both *in vivo* and *in vitro*. The vaccine has a large range of animal hosts, including mice, dogs, foxes, and skunks, either by parenteral or oral route. In all of these animals, the vaccine induces protective VNA against rabies virus infection (Charlton, Artois, Prevec, Campbell, Casey, Wandeler, & Armstrong, 1992; Prevec, Campbell, Christie, Belbeck, & Graham, 1990). In terms of the efficacy of ONRAB® and V-RG in striped skunks and raccoons, there is no significant difference in the rate of seroconversion (Fehlner-Gardiner, Rudd, Donovan, Slate, Kempf, & Badcock, 2012). This vaccine also has a high level of safety for many wild animals, including targeted or non-targeted species, such as eastern cottontail rabbits, wood rats, eastern wild turkeys, Virginia opossums, and fox squirrels (Fry, VanDalen, Duncan, & VerCauteren, 2013; Knowles, Nadin-Davis, Sheen, Rosatte, Mueller, & Beresford, 2009).

The third kind of oral rabies vaccine is the SAG family of vaccines. It contains two vaccines, SAG-1 and SAG-2, which are licensed in Europe, where more than 80% of rabies

cases are reported in red foxes. Having failed in controlling the number of foxes by some conventional methods like culling or trapping to control the spread of rabies, oral vaccination was then introduced to control rabies in the wild (G. M. Baer, Abelseh, & Debbie, 1971). Proven to be effective in 1969, the vaccine has been widely used since 1977 in Europe. SAG-1 and SAG-2 are selected from the SAD Berne strain with some mutations of the G protein (Schumacher, Coulon, Lafay, Benejean, Aubert, Barrat, Aubert, & Flamand, 1993). In SAG-2, the mutations make the strain genetically stable and apathogenic for adult mice, cats, dogs and foxes (Leblois, Tuffereau, Blancou, Artois, Aubert, & Flamand, 1990; Schumacher, Coulon, Lafay, Benejean, Aubert, Barrat, Aubert, & Flamand, 1993). Also, the immune efficiency and safety of the SAG-2 vaccine have been tested extensively in many animal species, including dogs, foxes, raccoons, and even skunks (Bingham, Schumacher, Aubert, Hill, & Aubert, 1997; Fekadu, Nesby, Shaddock, Schumacher, Linhart, & Sanderlin, 1996).

### **DNA vaccines**

The DNA vaccine is a radically novel approach to vaccination. It directly introduces a plasmid containing the antigen-encoding DNA sequence to appropriate tissues to produce target antigens, which will induce immune responses. The DNA vaccine has many advantages over conventional vaccines. With improved stability, it not only induces both B- and T-cell responses, but it also lacks the infectious agent. The first DNA vaccine for rabies was developed in 1994 (Xiang, Spitalnik, Tran, Wunner, Cheng, & Ertl, 1994). When animals were immunized with one dose of DNA expressing rabies virus G protein, only slow and modest VNA was induced, which is much lower than one-dose of the conventional rabies vaccine (Perrin, Yacob, & Tordo, 2000). However, after repeated



immunization, the VNA responses could be improved (Perrin, Yacob, & Tordo, 2000). In animal experiments in cats and dogs, with a moderate dose, the DNA vaccine can protect the animals from rabies virus challenge (Lodmell, Ewalt, Parnell, Rupprecht, & Hanlon, 2006; Osorio, Tomlinson, Frank, Haanes, Rushlow, Haynes, & Stinchcomb, 1999). Due to the slow immune response to DNA vaccines, it has been excluded from post-exposure use. One of the barriers that prevents the DNA vaccines from efficient antigen production *in vivo* is a lack of DNA uptake by cells at the inoculation site.

### **Live RABV vaccines**

The development of reverse genetics has resulted in generating more potent and safer modified-live rabies vaccines, by modifying viral elements responsible for pathogenicity and immunogenicity. Now, there are two types of live rabies vaccines, live-attenuated rabies vaccines expressing multiple copies of the G protein and live recombinant rabies vaccines expressing cytokines or chemokines. Live-attenuated rabies vaccines expressing multiple G proteins are generated by introducing multiple copies of the G protein into the rabies virus genome, which makes the virus less pathogenic and more immunogenic (Faber, Faber, Papaneri, Bette, Weihe, Dietzschold, & Schnell, 2005b). On the other hand, the cytokine or chemokine expressing vaccines enhance immune responses by activating and recruiting mature B cells and dendritic cells (DC) into the peripheral blood and draining lymph nodes (Zhao, Toriumi, Wang, Kuang, Guo, Morimoto, & Fu, 2010).

### **Replication-deficient RABV-based vaccines**

Replication-deficient vaccines have been developed for rabies based on safety concerns. The replication-deficient rabies vaccines are constructed by deleting viral genes

that are responsible for viral replication or assembly. There are M-deleted ( $\Delta M$ ) and P-deleted ( $\Delta P$ ) single-cycle rabies virus vaccines. When cells are infected by  $\Delta P$  rabies virus, genome replication will not be observed (Shoji, Inoue, Nakamichi, Kurane, Sakai, & Morimoto, 2004). The M-deleted rabies virus loses the bullet shape of rabies virus (Mebatsion, Weiland, & Conzelmann, 1999b). All of the replication-deficient rabies vaccines have been tested in mice and showed very good efficacy and safety.

### **Inactivated vaccines**

Inactivated vaccine is the most widely-used rabies vaccine licensed and sold in the USA for domestic animals (National Association of State Public Health Veterinarians, 2011). There are thirteen monovalent rabies vaccines for dogs and cats, two for ferrets, and some other vaccines for other species. For multivalent vaccines, there are three for dogs, cats, and horses, respectively. All of the licensed vaccines can be used interchangeably as either primary or booster vaccines. Like all other vaccines, rabies vaccines have some adverse reactions, including injection site swelling, fever, shivering, fatigue, vomiting, and hypersensitivity (Frana, Clough, Gatewood, & Rupprecht, 2008). The inactivated rabies vaccine from international companies, such as Intervet and Merial, are mostly used, which are all products from the developed countries. For the developing countries in Asia and Africa, the inactivated vaccines need to be imported and are thus too expensive.

### **Rabies reservoirs**

From all over the world, the most important reservoir of rabies is still the dog. But the situation is different between the developed countries and the developing countries. In the developed countries, wild animals are the main reservoir for rabies. In the United States,

since the 1950s, raccoons have been an important reservoir for the spread of rabies in the southeastern region (Finnegan, Brookes, Johnson, Smith, Mansfield, Keene, McElhinney, & Fooks, 2002). In other parts of the US, the skunk is an important reservoir of three strains of rabies virus (Finnegan, Brookes, Johnson, Smith, Mansfield, Keene, McElhinney, & Fooks, 2002). In 2006, about 92% of rabies cases were from wild animals, such as raccoons, bats, foxes, and skunks (Blanton, Hanlon, & Rupprecht, 2007). In Europe, the main reservoirs for classic RABV remains raccoon dogs and red foxes. In addition, the bat is also a principal reservoir (Chautan, Pontier, & Artois, 2000). In general, the control of rabies has been very successful.

However, in the developing or undeveloped countries, rabies cases mostly come from domestic dogs. In China, there are about 100 million dogs. It is reported that about 4.13%–17.82% of asymptomatic dogs in Sichuan and Hebei provinces are positive for rabies virus (Hu, Tang, Tang, & Fooks, 2009). In rural areas, the high cost of vaccination and poor awareness of rabies contribute to the low vaccination coverage of dogs for rabies. It was shown that only few dogs were vaccinated in 2 of 16 villages in Shandong Province (Hu, Tang, Tang, & Fooks, 2009). In rural areas, most dogs are not leashed and always roaming in these regions, thus increasing the risk of rabies exposure (Hu, Tang, Tang, & Fooks, 2009). Cats, domestic livestock, and wild animals are also important reservoirs. A similar situation also exists in India. There are approximately 25 million dogs, and about 80% are stray, unowned, and unprotected (Menezes, 2008). Since the stray dogs move around and are unrestricted, it is very difficult to catch them for vaccination.

## **Difficulties and problems in rabies vaccination**

According to the Compendium of Animal Rabies Prevention and Control, a booster vaccine needs to be given one year after the initial vaccination, regardless of the age of the animal. After that, booster vaccinations should be given annually or triennially as per local law (<http://www.rabiesaware.org/>). In the developed countries, domestic dogs and cats are immunized routinely, but require multiple vaccinations in their life. Usually, dogs are immunized initially at 3 months of age and then one year later. Subsequent booster immunization is carried out every one or three years depending on the vaccine used. Multiple vaccinations in animals not only add cost to the owners, but may also induce adverse reactions (for example, feline sarcoma). However, in the developing countries, routine vaccination of dogs is not carried out due to limited resources and a large population of stray dogs (self-sustaining reservoir). Multiple vaccinations of stray dogs are almost impossible due to problems of identification and capture.

Concerns about possible adverse effects from annual vaccination have prompted debate across various pet communities and experts to reanalyze the vaccine protocols and to extend the revaccination intervals. Commercial vaccine producing companies perform studies on the duration of immunity (DOI) to ensure the minimum DOI (minDOI) for their products to guarantee that their products convey immunity for a minimum period of time. However, the maximum DOI (maxDOI) is not often carried out due to the cost associated with long-term studies. There are no experimental or epidemiological data to support that the annual or biennial administration of 3-year vaccines is better (National Association of State Public Health, Compendium of Animal Rabies, Control, Brown, Slavinski, Ettestad, Sidwa, & Sorhage, 2016).

It is well known that rabies vaccination is the best way to control rabies. However, when we use the vaccine, there are also some adverse effects that may do harm to animals. In a report conducted by the Center for Veterinary Biologics (CVB), nearly 10,000 cases of an adverse event have been reported for rabies vaccines during a three-year period (Frana, Clough, Gatewood, & Rupprecht, 2008). Among these cases, about 65% are related to dogs (Frana, Clough, Gatewood, & Rupprecht, 2008).

When the vaccine is administered to dogs, in rare cases, it can lead to a deadly reaction known as anaphylaxis. Anaphylaxis can lead to symptoms as severe as a shock, cardiac failure, and respiratory failure. However, not all adverse reactions occur immediately. It can take as long as a few months for the dog to have a reaction to the vaccination. Reactions can be mild, such as skin disease or an allergic reaction near the injection site, but they can be serious.

In addition to the adverse effects that are caused by the vaccine reagent itself, there is also the possibility that the vaccines can be contaminated with other viral agents, which can result in significant untoward effects (Tizard, 1990). From 1940 to 1946, a contaminated rabies vaccine caused 40 cases of human encephalomyelitis in California (Pait & Pearson, 1949).

## **Goals and objectives**

The main objective of this study is to evaluate the protective efficacy of rabies vaccines in dogs that are vaccinated six and eight years previously. Immunological correlates have also been investigated by evaluating the immune responses, particularly the production of VNA and induction of memory B and T cells, and activation of DCs after

challenge. Successful completion of this study will provide evidence for extending the current 3 year rabies booster vaccination to 6 or 8 years.

## **CHAPTER 2**

### **METHODS**

#### **Materials**

Personal protective equipment (PPE) was needed for dog observation, including N95 respirator (3M), face shield (AlphaProTech), bonnet (Uline), disposable coverall (Tyvek), boot cover (Uline), and nitrile examination gloves (Ansell).

Phosphate-buffered saline (PBS) (1×) consisted of 8g NaCl, 0.2g KCl, 1.44 g Na<sub>2</sub>HPO<sub>4</sub>, 0.24 g KH<sub>2</sub>PO<sub>4</sub>, brought to a final volume of 1 L in deionized water.

TAE buffer (50×) was made by dissolving 242g of Tris base in water, 57.1 mL glacial acetic acids, 100 mL of 500mM EDTA (pH 8.0) solution and bringing the final volume to 1 L.

#### **Viruses and animals**

A canine New York (NYC) strain RABV, a challenge virus originally isolated from a rabid dog and adapted in foxes, was obtained from USDA. The lethal dose of NYC was used in our study to challenge the dogs. The NYC virus was stored at -80°C.

The dogs were all female beagles, which had been raised at Ridgman Farms. INC., Blue Mounds, WI. A group of five dogs, which had been vaccinated in the year 2007 using Merial rabies vaccine, and another group of five dogs, which had been vaccinated in the year 2009 using the Intervet rabies vaccine was included in the study. Five, unvaccinated, age-matched dogs were used as controls.

## **DNA**

The primer for confirmation of rabies virus was generated from the N gene, and it is 127bp. The sequence of the forward primer, Primer F, is ATCTCACCGCAAGGGAAGC, 5'-3'. The sequence of the reverse primer, Primer R, is AGT GAA CGG AAG TGG ATG AAA T, 5'-3'.

## **Dog infection and observation**

Dogs were infected I.M. with New York (NYC) street rabies (1000 LD50) by direct inoculation into the left hemisphere of the temporalis muscle. Challenged dogs were observed at least once a day prior to challenge and three times a day for 90 days after challenge. The humane endpoint of the study was considered the appearance of hind limb paralysis of one or both limbs, and the experimental endpoint of the study was determined on the basis of observed clinical signs.

## **Blood collection and lymph node aspiration**

Blood samples were collected at 0, 4, 12 and 26 dayspost-challenge for the quantification of VNA and immune responses. For blood collection, dogs were pretreated with Acepromazine (2 mg/kg). When the dogs calmed down, they were injected with Telazol (100 mg/mL) at 4 mg/kg using a pole-syringe. Once unconscious, the dogs were transported to the procedure room. Ten mL of blood was collected from the jugular vein. At the indicated time points, whole blood was collected from the dogs, allowed to clot overnight at 4°C, and spun down at 3,000 rpm for 10 min. Subsequently, serum was isolated for analysis.



Lymph node samples were collected at 4, and 12, days post-challenge for analysis of immune responses. Under anesthesia, the left mandibular lymph node was aspirated with a 3 mL syringe with a 20G needle punctured into the lymph node and loaded into a 1.5 mL Eppendorf tube containing 1 mL of  $1 \times$  PBS solution.

### **Brain extraction**

Once the rabid dogs reached human end points or the surviving dogs reached the 90-day study period, dogs were humanely euthanized with 5 mL euthanasia solution (Beuthanasia-D special). Brains were extracted for the detection of RABV antigen. Briefly, as soon as the dogs reached the end-point, the dogs were euthanized with euthanasia solution at 0.5 mL/kg. After the cessation of heartbeat, the dog was beheaded, and the skull was opened using a skull breaker. Then, the brain was carefully extracted using forceps. Then, extracted brain was washed thrice with sterile PBS and cut into two halves, one half was fixed in 10% formalin, and the other half was frozen in  $-80^{\circ}\text{C}$  for virus isolation.

### **Rabies virus genome confirmation by PCR**

#### **Extraction of RNA**

In order to detect the presence of RABV genome in the brain by PCR, 50mg brain stem was collected from each sample. Then, 1 mL TRIzol® Reagent was added to the sample, ground with a pestle and incubated for 5 min at room temperature for complete dissociation of the protein complex. This homogenate was added to 0.2 mL of chloroform. Then, the mixture was vortexed for 15s and incubated for 3 min at room temperature. After centrifuging at  $12,000 \times g$  for 15 min at  $4^{\circ}\text{C}$ , the sample was separated into three phases,

collecting the aqueous phase of the sample by pipetting the solution out. The aqueous phase was then added to 0.6 mL of 100% isopropanol, incubated at room temperature for 10 min, and centrifuged at  $12,000 \times g$  at  $4^{\circ}\text{C}$  for 10 min. The RNA pellet was left at the bottom of the tube after removal of the supernatant. 75% ethanol was used to wash the pellet, which was then briefly vortexed and centrifuged at  $7500 \times g$  for 5 min at  $4^{\circ}\text{C}$ . The RNA pellet was air-dried for 5 min. Then, 30  $\mu\text{L}$  RNase-free water was added to resuspend the RNA. The RNA sample was incubated in a heat block at  $55^{\circ}\text{C}$  for 10 min and then stored at  $-80^{\circ}\text{C}$ .

### **cDNA synthesis**

In order to synthesize cDNA of the RNA, RT-PCR was performed in two steps in a 25  $\mu\text{L}$  volume. In the first step, 500ng of isolated RNA was added to the cDNA mixture containing 1  $\mu\text{L}$  oligo dT<sub>40</sub> (10  $\mu\text{M}$ ), and 1  $\mu\text{L}$  10mM dNTP and brought up to 13  $\mu\text{L}$  using sterile distilled water. The mixture was incubated at  $65^{\circ}\text{C}$  for 5min, and on ice for 2 min. Then, 4  $\mu\text{L}$  of  $5 \times$  First-Strand buffer, 1  $\mu\text{L}$  0.1M DTT, 1  $\mu\text{L}$  RNase inhibitor (Roche), and 1  $\mu\text{L}$  Superscript II (Invitrogen) were added to the mixture. The sample was incubated at  $55^{\circ}\text{C}$  for 60 min and  $70^{\circ}\text{C}$  for 15 min. After the completion of incubation, the cDNA products were used for PCR confirmation using RABV specific primers.

### **PCR**

Distilled water was added to the mixture of 0.5  $\mu\text{L}$  Ex Taq (Invitrogen), 2  $\mu\text{L}$  Primer F, 2  $\mu\text{L}$  Primer R, 4  $\mu\text{L}$  dNTP, 4  $\mu\text{L}$   $10 \times$  buffer, 4  $\mu\text{L}$  cDNA, and brought up to 40  $\mu\text{L}$ . The PCR reaction was run on an Express Gene gradient cycler (Deville Scientific Inc.).

The PCR program used was as follows:

- $94^{\circ}\text{C}$  5 min

35 cycles of:

- 94°C 30s, denaturation
- 50°C 30s, annealing
- 72°C 30s, extension

Final extension:

- 72°C 5 min

### **Detection of nucleic acids**

Loading buffer was added to the PCR products and run on a 1% agarose gel. 0.5 µg of a 1kb DNA ladder (Invitrogen) was run as a standard.

### **Flow cytometry**

The blood and lymph node aspirates were collected prior to challenge and at 4 and 12 dpc. Peripheral blood mononuclear cells (PBMC) were isolated from 10mL of blood using Ficoll Histopaque solution (Sigma-Aldrich). The isolation was performed according to the protocol. Briefly, 5mL of whole blood anticoagulant was gently mixed with 5mL of 1× PBS (pH=7.4). The 10mL diluted blood solution was overlaid on top of 5mL Ficoll-paque Plus in a 15mL centrifuge tube at room temperature and centrifuged at  $400 \times g$  for 20 min at 20°C. After the centrifugation, the granulocytes, platelets, and RBCs were pelleted to the bottom of the tube and the PBMC floated over the Ficoll-Paque PLUS. The PBMCs were carefully aspirated from the Ficoll-plaque interface and put into a new 15mL centrifuge tube. Five mL of 1 × PBS were added to the tube containing PBMCs, mixed gently, and centrifuged at  $200 \times g$  for 10min at 20°C. The supernatant was discarded, and 1 × PBS was added to 15 mL to resuspend the cells, and the tube mixed gently. The tube was centrifuged at  $200 \times g$  for 10min at 20°C. The supernatant was discarded, and the cells were resuspended using the remaining 1 × PBS solution.

Then, cells were stained with CD3, CD4, CD8, CD11c, CD19, CD25, CD27, CD40, CD62L, CD86, and CCR7 antibodies, and isotype control. For surface staining, 100 $\mu$ L of diluted antibodies were added to each well and incubated for 30min in the dark at room temperature. The plate was centrifuged at 400 $\times$ g for 5min, and the supernatant discarded. Then the cells were washed twice with washing buffer. One hundred  $\mu$ L 2% formaldehyde (diluted with PBS) was added and the cells incubated for 30min at 4°C. The cells were washed twice using the washing buffer and resuspended with 300 $\mu$ L staining buffer, then transferred into 5mL flow cytometry tubes.

Data collection and analysis were performed using a BD LSR-II flow cytometer, BD FACSDiva software (BD Pharmingen), and FlowJo software (TreeStar, San Carlos, CA).

### **RFFIT (Rapid Fluorescent Focus Inhibition Test)**

Blood samples were collected for the measurement of VNA by the RFFIT. Briefly, 50  $\mu$ L of serum in serial three-fold dilutions, were added to a 96-well plate (Nalge Nunc International, Rochester, NY). Fifty FFD50 (50% Focus Forming Dose) of CVS-11 was added to every well and incubated at 37°C for 90 min. NA cells ( $10^5$  cells) were added to each well and incubated in a CO<sub>2</sub>-controlled incubator at 37°C for 20 hours, fixed with 80% ice-cold acetone, and stained with FITC-conjugated anti-RABV N antibodies (Fujirebio Diagnostics, Inc.) at 37°C for 1 hour. In all wells, twenty fields were observed under a fluorescent microscope with a 10 $\times$  objective, and the 50% endpoint titers were calculated based on the Reed-Muench formula. The values were compared with reference

serum (supplied by the National Institute for Biological Standards and Control, Herts, UK) and normalized to international units (IU/mL). The lower limit of detection was 0.1 IU/mL.

### **Ethics statement**

This study was approved by the University of Georgia's Institutional Animal Care and Use Committee. The University Research Animal Resources unit of The University of Georgia is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International (AAALAC-I).

### **Statistical analysis**

Statistical significance of the differences between groups was tested with student's T-test, with \* indicating a p value < 0.05, \*\* indicating a p value < 0.001, and \*\*\* indicating a p value < 0.0001 using GraphPad prism software.

## **CHAPTER 3**

### **RESULTS**

#### **Clinical observations**

Clinical signs were observed for evaluation of the process of rabies infection. Among the 15 dogs challenged with NYC street rabies virus, 5 unvaccinated dogs showed characteristic signs of rabies. In these 5 dogs, all were euthanized, 2 dogs reached the humane endpoint at 13 and 15 days post-challenge (dpc), and the remaining 3 dogs reached the endpoint by 18 dpc. Early signs included subtle changes in behavior, such as quietness, drowsy eye contact, dilation of pupils, and loss of appetite. Later signs consisted of restless, agitation, profound salivation, and difficulty in swallowing or drinking, poor coordination, trembling, and retching. For the dogs vaccinated 8 years ago, 4 out of 5 showed characteristic signs of rabies and reached the humane endpoint at 16, 17, 19, and 25 dpc, respectively. For the dogs vaccinated 6 years ago, only 1 out of the 5 showed characteristic signs of rabies, at 17 dpc. All of the surviving dogs in the vaccinated groups did not show any clinical signs of rabies during the 90-day observation period. All of the surviving dogs were euthanized after the 90-day observation period. Blood samples were collected and brains extracted for analysis.

#### **VNA responses in the serum**

VNA titer was measured to evaluate the immune responses induced in all dogs. All of the serum samples were collected at four time points during the observation, prior to

challenge and at 4, 12, and 26 dpc or at the time of euthanasia for surviving dogs. RFFIT analysis was performed on all samples to quantify the VNA. As summarized in Table 1, none of the dogs from any of the groups had a VNA titer equal to or higher than 0.5 IU/mL prior to challenge, except one of the dogs that had been vaccinated 8 years ago, which had serum VNA titer of 0.5 IU/mL. The unvaccinated dogs had an average serum VNA of 0.03, 0.056, 0.2, and 0.43 IU/mL at 0, 4, 12 dpc and endpoints, respectively. Whereas, the dogs that had been vaccinated 8 years ago had average serum VNA of 0.13, 0.29, and 1.77 IU/mL at 0, 4, and 12 dpc, respectively. Four dogs that died from rabies had an average end-point serum VNA of 3.13 IU/mL. However, the one dog that survived the lethal challenge had serum VNA of 0.5, 1.14, 7.79, 23.38 and 3.8 at 0, 4, 12, 26 dpc and at the endpoint, respectively. Six year-vaccinated dogs had an average serum VNA of 0.06, 0.13, 7.36, 10.42, and 3.32 IU/mL at 0, 4, 12, and 26 dpc and at the endpoint, respectively (Fig.1). In addition, one of the 6 year-vaccinated dogs that succumbed to lethal rabies challenge had an endpoint VNA of 5.9 IU/mL.

Analysis of immune responses in dogs that survived the lethal challenge showed significantly higher levels of serum VNA at all time points, except the end points. Dogs that survived the lethal challenge had an average VNA of 0.16, 0.34, 8.52, 13.01 and 2.90 at 0, 4, 12, and 26 dpc and at the endpoint, respectively vs. the dogs that had succumbed to rabies that had an average VNA of 0.03, 0.06, 0.4, and 2.1 IU/mL (Fig.2).

### **Cellular immune responses to lethal rabies infection in dogs that had been vaccinated**

Further, to evaluate the cellular immune responses in all dogs, the number of CD4<sup>+</sup> Th cells, CD8<sup>+</sup> cytotoxic T cells, memory T cells, B cells and activated DCs are measured

by flow cytometry. All of the blood and lymph node samples collected prior to challenge and at 4, 12, and 26 dpc or at the time of euthanasia for surviving dogs were analyzed by flow cytometry for the quantification of the number of CD4<sup>+</sup> Th cells and CD8<sup>+</sup> cytotoxic T cells, memory T cells, B cells and activated DCs. At 4 dpc, a significant number of memory B cells were detected in dogs that were vaccinated (6 and 8 years ago) than the unvaccinated dogs, but only in lymph nodes and not in blood (Fig. 3G). No significant differences were observed in other cell types between vaccinated and unvaccinated controls in blood or in the lymph nodes at 4 dpc.

However, in the blood at 12 dpc, significant differences in the number of memory T cells and activated DCs were detected only in dogs that had been vaccinated 6 years ago than the unvaccinated or dogs that had been vaccinated 8 years ago. Whereas, in the lymph node, significantly more activated DCs were detected in 6 year-vaccinated dogs than the unvaccinated or dogs that had been vaccinated 8 years ago (Fig.4). Thus, cellular immune responses at 12 dpc has the best correlates with protection against lethal rabies infection.

### **Protection against lethal rabies challenge**

Survival rates were calculated to evaluate the protection efficacy. The three groups of dogs were observed for 90 days post rabies virus challenge. As expected, all of the unvaccinated dogs died from rabies. However, in accordance with the immune response data, 80% of dogs that had been vaccinated 6 years ago were protected, and only 20% of the dogs that were vaccinated 8 years ago survived the lethal challenge, until the end of 90 days observation period without any clinical signs of rabies infection (Fig.5).



### **Rabies virus genome confirmation by PCR in the brain**

In order to confirm that the dogs had all succumbed to rabies infection and there was no virus present in surviving dogs, PCR was performed on the brain samples of all dogs. Brain samples, collected when the dogs are euthanized, were analyzed for the presence of the rabies viral genome (N-gene) by RT-PCR. As presented in figure 6, all of the dogs that died from rabies showed positive results for the presence rabies N gene (127bp) in the respective brain samples and all the survival dogs showed negative results. Rabies virus B2c was used as a positive control (Fig.6).

## **CHAPTER 4**

### **DISCUSSION**

Rabies is an ancient disease but still causes more than 59,000 human fatalities each year in the world, mostly in Africa and Asia (WHO, 2013). Although the disease is preventable through timely pre-and post-exposure vaccination, death is inevitable once clinical signs appear (Alan C. Jackson, 2013). Rabies is transmitted to humans through animal bites, most commonly by dogs. Thus, controlling or eliminating dog rabies is always the priority. Mass vaccination of dogs has resulted in the control or elimination of canine rabies in many countries in the Americas and Europe (Cliquet & Aubert, 2004; Lontai, 1997). As a consequence, human rabies has declined dramatically during the past 50 years (Hampson, Coudeville, Lembo, Sambo, Kieffer, Attlan, Barrat, Blanton, Briggs, Cleaveland, Costa, Freuling, Hiby, Knopf, Leanes, Meslin, Metlin, Miranda, Muller, Nel, Recuenco, Rupprecht, Schumacher, Taylor, Vigilato, Zinsstag, Dushoff, & Par, 2015). In countries where canine rabies vaccination is not practiced routinely, rabies is still endemic, causing human fatalities.

According to the American Animal Hospital Association (AAHA) Canine Vaccine Task Force guidelines, the first dose of rabies vaccine should be given to dogs between 12 to 16 weeks of age. A booster dose is given one year later. Subsequent vaccination should be given every one or three year(s) depending on the vaccines given or as per local law (<http://www.rabiesaware.org/>). In the USA and many other countries, this regime of rabies vaccination is mandated by laws and regulations (WHO, 2005b, 2013). Concerns about

possible adverse effects from annual booster vaccination have prompted debate across various pet communities and experts to reanalyze the vaccine protocols and to extend the revaccination intervals (Frana, Clough, Gatewood, & Rupprecht, 2008). Multiple vaccination schedules also present problems in the developing countries, where rabies is still endemic and causes huge human and financial losses. Firstly, the cost of the vaccine is a huge burden for those countries, since most rabies vaccines used in the developing countries are imported from the developed countries; the price is sometimes unaffordable. Secondly, there are a large number of stray dogs, and it is difficult to catch these dogs for primary and subsequent booster vaccinations (Totton, Wandeler, Zinsstag, Bauch, Ribble, Rosatte, & McEwen, 2010). Therefore, rabies vaccines capable of inducing long-term protective immunity will not only reduce the cost and side effects associated with vaccines, but also reduce the burden for immunizing stray dogs. In the present study, the duration of immunity after a single shot was evaluated in dogs, and it was found that dogs that had received a single shot of rabies vaccine at three months of age could be protected for up to six years. Our results show that the single shot of rabies vaccine can protect dogs for up to six years.

Previous studies by Aubert have shown that the DOI against rabies is five years by challenge and seven years by serological studies (Aubert, 1992). In their study, none of the dogs had detectable antibodies before challenge; however, more than 90% of the dogs survived (Aubert, 1992). Likewise, most of our dogs did not have detectable VNA prior to challenge in the group immunized 6 years ago, yet 80% of the dogs survived. Evaluation of long-term protective immune responses in our study indicates that the dogs that had been vaccinated 6 and 8 years ago are capable of inducing memory B cell responses and a high

level of VNA production. However, only 20% of the 8 years vaccinated dogs were protected against lethal challenge, indicating the maximum duration of protection could only be up to 6 years.

Further, this is the first report on memory B and T cells in rabies-challenged dogs. Evaluation of cellular immune responses shows that the dogs that had been vaccinated six and eight years ago are capable of inducing the early generation of memory B cells than the unvaccinated dogs. This clearly indicates that the memory recall immune responses are not greatly affected by the duration of vaccination *per se*. However, the early induction of memory B cells was maintained only in the dogs immunized 6 years ago, along with memory T cells and activated DCs. Further, the maintenance of memory B cells contributed to the significant production of VNA and thereby better protection against lethal challenge. Whereas, in the 8-year vaccinated dogs, early activation of memory B cells quickly waned off at 12 dpc, thus, they were unable to mount protective immune responses against lethal challenge. It is possible that the insufficient and unsustainable immune responses observed in the eight year-vaccinated dogs could be due to age-related immune exhaustion, rather than due to the longer duration of immunization. Various studies have shown aging associated changes in cellular and humoral immunity in humans, as well as in dogs (J. Wu, Li, Liu, Zhang, Peng, Feng, Li, Wang, Liu, Li, & Liu, 2012). Decreased activity of Th cells, CTLs, and NK cells, and tumoricidal activities have been reported in aged dogs (J. Wu, Li, Liu, Zhang, Peng, Feng, Li, Wang, Liu, Li, & Liu, 2012). Further studies are warranted to rule out the age-related immune responses. However, the lethal challenge in six and eight-year rabies-vaccinated dogs indicate that the duration of protective immunity can be up to six years.

The long DOI for rabies protection could greatly benefit societies in terms of controlling canine and human rabies all over the world. For developed countries, the six-year-long protective period helps to reduce the frequency of booster vaccinations. Along with the reduction of revaccination, time and money will be saved and adverse reactions reduced. Our studies will help regulatory agencies to consider whether to extend the booster vaccination from three years to six years. For developing countries, the large number of stray dogs is a tough problem (Menezes, 2008). Due to animal welfare and religious concerns, the population of stray dogs is not easy to control (Menezes, 2008). Thus, vaccination needs to be performed to control rabies among the stray dogs. Because of the long DOI of the current vaccines, vaccination of stray dogs can become easier without the need for subsequent booster vaccinations. When the ratio of vaccinated stray dogs reaches 70%, the herd immunity is formed (Thulke & Eisinger, 2008). Thus, the six-year-long protective period provides a means to control rabies in the stray dog population. The control of rabies in stray dogs will definitely decrease the cases of human rabies since most of the human cases are caused by dog bites in developing countries (Ichhpujani, Bhardwaj, Chhabra, & Datta, 2001).

The current data have demonstrated that protective immunity can last up to 6 years after a single shot of rabies vaccine. Developed countries will benefit in the cost of raising pets and animal welfare, while developing countries can have a better control of rabies by vaccinating stray dogs; thus, human rabies cases will decrease.

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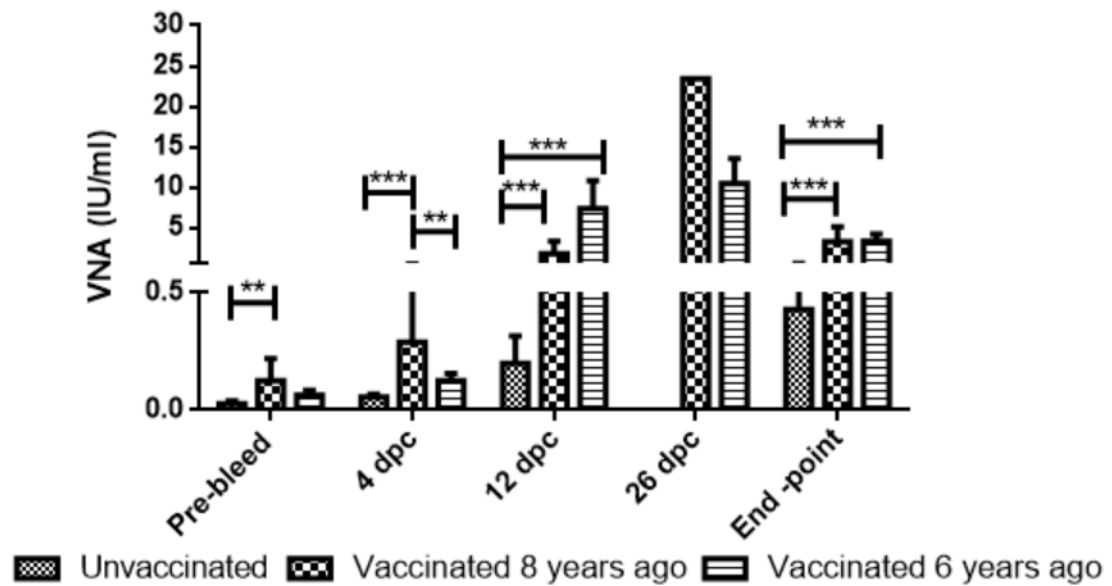
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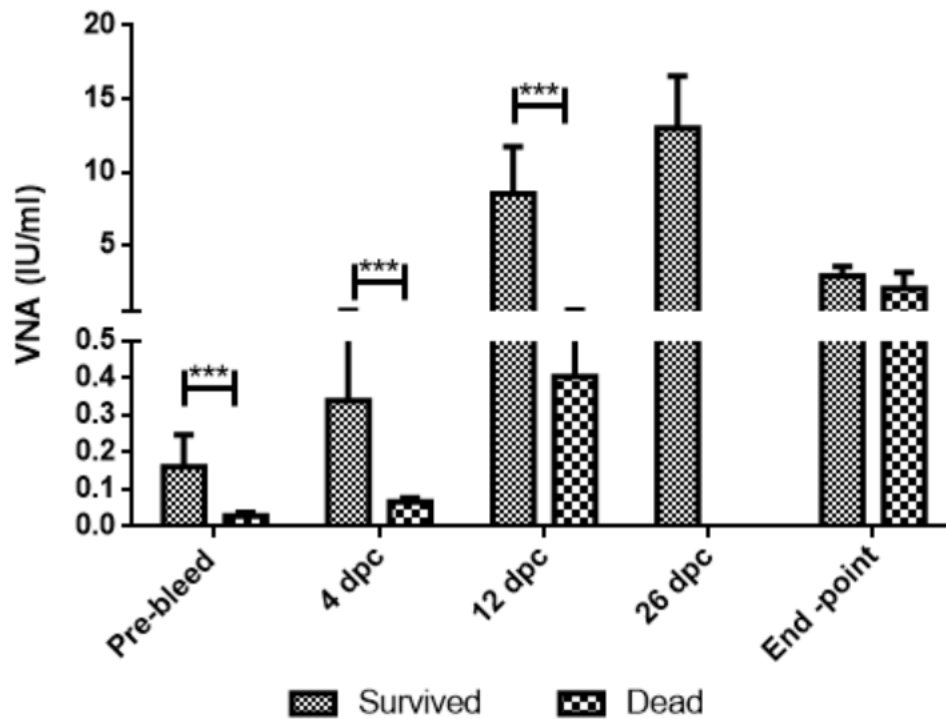
immunogenicity by inducing innate immunity and recruiting dendritic cells and B cells. J Virol, 84(18), 9642-9648. doi:10.1128/JVI.00326-10

Table 1: VNA titer (IU/mL) in the serum.

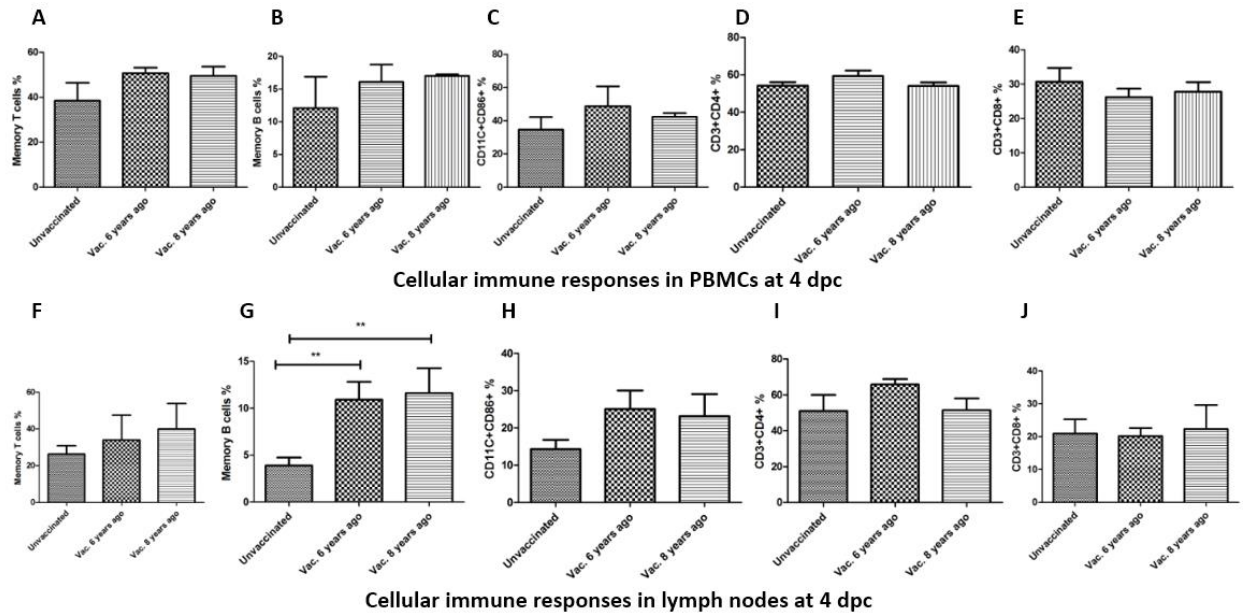
	Dog ID	Pre-challenge	Day 4 post-challenge	Day 12 post-challenge	Day 26 post-challenge	Endpoint	Comments
Unvaccinated Control	BXO	0.07	0.07	0.1	-	0.38	Dead/18dpc
	FTQ	0.02	0.02	0.66	-	1.14	Dead/18dpc
	IZS	0.03	0.03	0.13	-	0.38	Dead/15dpc
	SQO	0.01	0.01	0.07	-	0.22	Dead/18dpc
	XLI	0.01	0.01	0.04	-	0.03	Dead/13dpc
Rabies vaccination 8 years ago	CKS	0.01	0.06	0.13	--	10.26	Dead/17dpc
	SIS	0.01	0.13	0.22	-	0.29	Dead/16dpc
	OOQ	0.01	0.02	0.5	-	1.5	Dead/19dpc
	ORQ	0.1	0.1	0.22	-	0.5	Dead/25dpc
	EQS	0.5	1.14	7.79	23.38	3.8	Alive
Rabies vaccination 6 years ago	ADE	0.04	0.07	17.77	5.92	2.2	Alive
	AEE	0.07	0.22	3.42	17.77	5	Alive
	KVE	0.07	0.17	0.13	4.5	1.3	Alive
	PUE	0.13	0.1	13.5	13.5	2.2	Alive
	YXE	0.01	0.07	1.97	-	5.92	Dead/17dpc



**Figure 1. Comparison of VNA levels at different time points.** Dogs (groups of 3) were infected i.m. with 1000 LD50 NYC RABV. Serum was collected on 0, 4, 12, and 26 dpc and at the endpoint. RFFIT analysis was performed on all samples to quantify the VNA. Data were analyzed by Student's T-test. Asterisks indicate significant differences (\* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001) between groups.

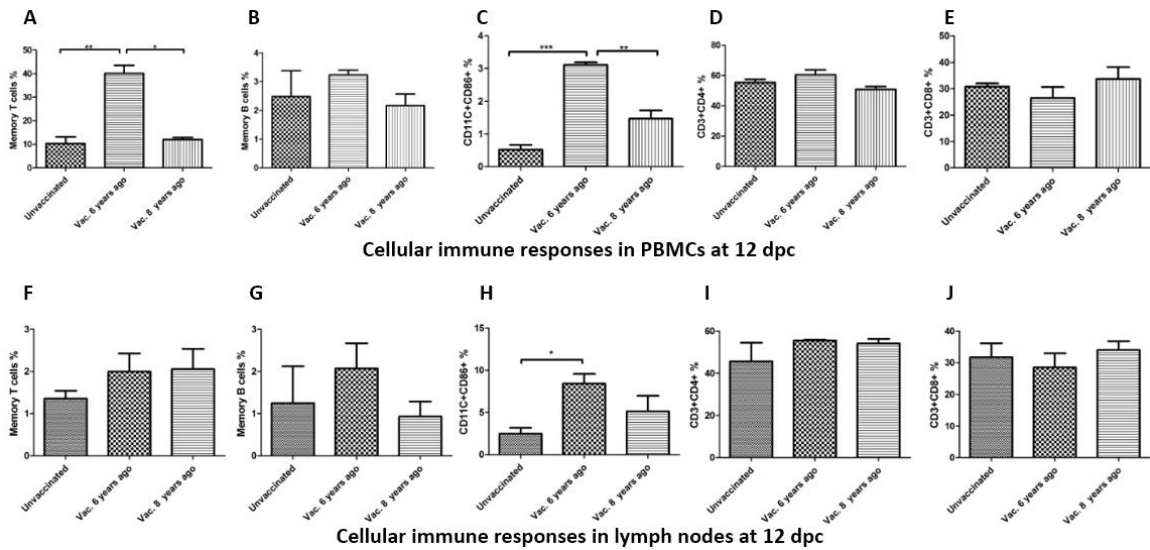


**Figure 2. Immune responses between dogs that had survived vs. succumbed to lethal rabies challenge at different time points.** Dogs (groups of 2) were infected i.m. with 1000 LD50 NYC RABV. Serum was collected on 0, 4, 12, and 26 dpc and at the endpoint. RFFIT analysis is performed on all samples to quantify the VNA. Data were analyzed by Student's T-test. Asterisks indicate significant differences (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ) between groups.



**Figure 3. Cellular immune responses in blood and lymph nodes at 4 dpc.** Dogs

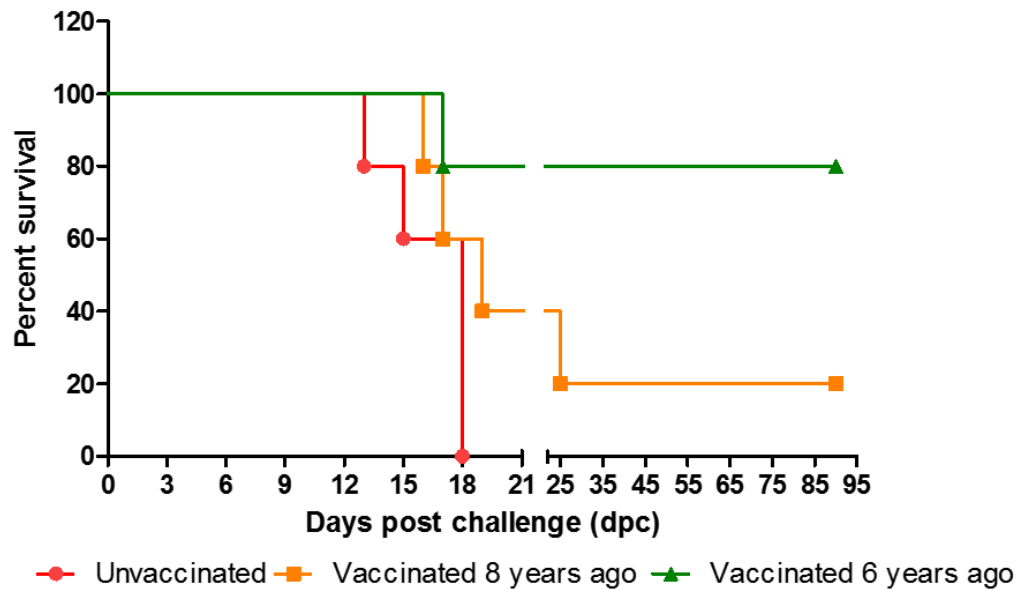
(groups of 3) were infected i.m. with 1000 LD50 NYC RABV. Blood and lymph node samples were collected at 4 dpc. Flow cytometry was performed on all samples for the quantification of memory T cells (**A, F**), B cells (**B, G**), activated DCs (**C, H**), CD4<sup>+</sup> Th cells (**D, I**) and CD8<sup>+</sup> T cells (**E, J**) in blood and lymph nodes. Data were analyzed by Student's T-test. Asterisks indicate significant differences (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ) between groups.



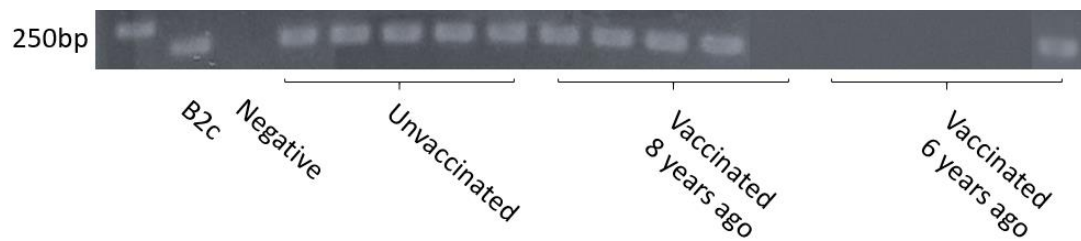
**Figure 4. Cellular immune responses in blood and lymph nodes at 12 dpc. Dogs**

(groups of 3) were infected i.m. with 1000 LD50 NYC RABV. Blood and lymph node samples were collected at 12 dpc. Flow cytometry was performed on all samples for the quantification of number of memory T cells (**A, F**), B cells (**B, G**), activated DCs (**C, H**), CD4<sup>+</sup> Th cells (**D, I**), and CD8<sup>+</sup> cytotoxic T cells (**E, J**) in blood and lymph nodes. Data were analyzed by Student's T-test. Asterisks indicate significant differences (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ) between groups.





**Figure 5. Efficacy of rabies vaccine in dogs.** Dogs (groups of 3) were infected i.m. with 1000 LD50 NYC RABV. Blood and lymph node samples were collected at 4 dpc. Infected dogs were observed for 90 days, and survivorship was recorded and analyzed.



**Figure 6. Rabies virus genome confirmation by PCR in the brain.** Dogs (groups of 3) were infected i.m. with 1000 LD50 NYC RABV. Brain samples were collected at the time when they succumbed to rabies or at the end of the observation period. PCR was performed to detect the presence of rabies virus. B2c strain was used as a positive control. Distilled water was used as a negative control.