ABSTRACT

LINDSAY STEIN
An immunohistochemical study of the distribution of rabies virus within the central nervous system of various mammalian species
Under the Direction of DR. CORRIE BROWN

In a retrospective study, immunohistochemistry was applied to 37 archival cases of rabies using a commercial polyclonal antibody. Thirteen different species were studied including: 3 dogs, 4 cats, 1 pig, 5 cattle, 3 horses, 1 llama, 7 skunks (*Mephitis mephitis*), 7 raccoons (*Procyon lotor*), 1 bat (*Myotis* sp.), 1 white-tailed deer (*Odocoileus virginianus*), 1 bobcat (*Lynx rufus*), 2 gray foxes (*Urocyon cinereoargenteus*), and 1 red fox (*Vulpes vulpes*). All cases had previously been diagnosed as rabies using histopathology and/or fluorescent antibody testing. The immunohistochemistry technique successfully detected the presence of rabies virus antigen in every case, and highlighted characteristic distributional differences throughout the brain for most species. The results showed the hippocampus as the best site for rabies detection in both dogs and cats. For cattle, the virus particles were most prominent in the brainstem, followed by the cerebellum. In horses, the cervical spinal cord and adjacent brainstem proved to be optimal sites for detecting rabies. In raccoons and skunks, positive labeling was widely dispersed and selection might be less important for these wildlife reservoir species. Immunohistochemistry should prove useful in enhancing the accuracy of rabies diagnosis through informed selection of brain segments when composite sampling is not feasible. This technique, which uses formalin-fixed tissue, has several advantages over fluorescent antibody testing which is performed on fresh tissue; specifically, it avoids any biosafety hazards in transport or in the laboratory. The widespread success of this commercial polyclonal antibody allows for rapid and reliable virus detection in any mammalian species, and immunohistochemistry shows great promise for becoming a universal test, especially for diagnostic laboratories in the developing world, where human rabies deaths are still prevalent.

INDEX WORDS: Diagnostic pathology, Immunohistochemistry, Lyssavirus, Neuropathology

AN IMMUNOHISTOCHEMICAL STUDY OF THE DISTRIBUTION OF RABIES VIRUS WITHIN THE CENTRAL NERVOUS SYSTEM OF VARIOUS MAMMALIAN SPECIES

by

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DEDICATION

This thesis, and all of the hard work put into research and writing, is dedicated to both of my parents, whose excitement and curiosity in my work sometimes exceeded my own. Such an achievement would not have been possible without their love and encouragement throughout all of my many pursuits. In the same ways in which they were my biggest fans on the soccer field, they have now become even greater enthusiasts of my academic involvements, and are the ones I strive to make proud the most. This is for my mom, whose reassurance and optimism is sure to lift my spirits whenever I feel defeated, and to my dad, whose valuable input and perspective always helps me refocus and regain confidence to keep working. I am only where I am today because of the high expectations my parents had for me, so that I, too would never set the bar too low for what I decided to take on. There is nothing more encouraging than knowing my parents always believe in what I aim to achieve, and that they are proud regardless of the results at the end of the day, as long as I simply gave it my best. For all the times they have been there alongside me through the many challenges I have endured, they deserve to share in all of my successes as well. No matter where my research and education takes me, or what things I decide to take on in the future, I know they will be behind me all of the way with their unfailing love. I cannot thank God enough for blessing me with the two of them.

ACKNOWLEDGEMENTS

The success of this research and completion of this thesis would not be possible without Dr. Corrie Brown, Dr. Raquel Rech, all the researchers in Dr. Brown's pathology lab, and Dr. Kevin Keel, who provided us with the wildlife cases to incorporate into our study. I must first acknowledge my faculty research mentor, Dr. Corrie Brown, and my reader, Dr. Raquel Rech, for both guiding me along in this process. They opened my eyes to the world of research and infectious diseases, and its many practical applications in a future in veterinary medicine. I want to thank Dr. Brown for taking me in as a freshman and inviting me to share in the limitless possibilities of her lab. She has truly fulfilled her title as a mentor, and her expertise in the field and heart for teaching and guiding students has made my experience as an undergraduate invaluable. I also have to thank Raquel, who introduced me to the fascinating research that she began and allowed me to become her rabies "partner in crime". Dr. Brown could not have made a better match of a graduate and undergraduate, and I will never forget all those late afternoons in the lab with Raquel, marveling at the beautiful staining of our slides. I must also thank Jian Zhang, our lab technician, and the other graduate students in the lab who were forced to put up with my undergraduate inexperience, endless questions, hectic hours, and lack of foreign diversity. For fully embracing me the moment I walked into the lab, never giving up on me, and teaching me all the tricks of the trade, I am so grateful. Everyone's enthusiasm, laughter, and passion for pathology quickly became as infectious as the diseases we studied. As I head into the future, I will never forget all of the great times in the lab, all that I learned in doing my research, and those who helped me along the way.

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CHAPTER 1
INTRODUCTION

Rabies is a neurologic disease characterized by acute, progressive, often fatal encephalitis.[1,3,20,21,27] It is probably the oldest known zoonosis, with its fatal effects being traced back more than 5,000 years.[10,20,21] Despite continued advancements in rabies control programs and research in the understanding of viral pathogenicity, rabies still remains the zoonosis with the highest case-fatality ratio with more than 55,000 human deaths per year.[5,7,20,21,22] It is estimated that one person dies from the disease every 15 minutes and another 300 individuals are exposed.[20] The main reservoirs of the virus include animals of the Order Carnivora and Chiroptera, but all mammalian species are susceptible at any time, and the disease is found on every continent except Antarctica.[2,14,20,26] While canine rabies has been greatly diminished in the United States and Latin America through large-scale vaccination programs, and even eradicated in some countries such as Great Britain, New Zealand, Australia and Japan, wildlife rabies continues to be maintained by certain reservoir species and constitutes more than 90% of all rabies cases reported each year in the US.[9,14,23,24,26,27]

Rabies virus is a member of the *Lyssavirus* genus of the family Rhabdoviridae. The bullet-shaped virions measure about 75 nm by 200 nm and contain one molecule of negative-sense, single-stranded RNA.[24,26,27] The viral genome contains five monocistronic genes that encode for five viral proteins. The glycoprotein appears to be very important for neurovirulence, and variation in this structure leads to different rabies virus variants.[20,23,24] The virus, which is eventually shed in the saliva of the host, is almost always transmitted through bite by an infected animal, though non-bite exposures can occur, such as inhalation of viral particles in a highly

infected bat cave, laboratory accidents, or organ transplants.[14,24,27] At the exposure site, the virus replicates in the muscle tissue and eventually binds to nicotinic acetylcholine receptors at the neuromuscular junction and enters the peripheral neurons.[14,20,23,26,27] From here, the virions travel in a retrograde manner along axons and across synapses to the central nervous system (CNS). Replication and viral dissemination occur widely throughout the CNS before the virus spreads centrifugally to the salivary glands and other tissues and organs.[3,7,14,23,26,27] Viral particles can be secreted in the saliva a few days prior to the onset of neurological signs, leading to the spread of rabies to other hosts.[3,14,20] The disease has a rapidly worsening clinical course that usually lasts only 3-10 days, and almost always ends with fatality.[7,14,21,23] There is no effective treatment for rabies in humans, but prompt postexposure prophylaxis (PEP) treatment will almost always prevent the disease from developing.[21,27]

There are no definitive or species-specific clinical signs for rabies. The most important signs are behavioral alterations,[21,23] but identifying these changes can be hard in many wildlife species, and even rabies in horses and cattle often goes undetected by livestock farmers.[17] When describing the clinical presentation of rabies virus, there are usually two different forms - the "furious" and the "paralytic" forms. Furious rabies victims display aggressiveness, destructive behavior, biting and agitation, while those individuals that develop dumb rabies show lethargy, stupor and paralysis with minimal aggression.[7,23,26] It is not possible to detect rabies infection during the incubation period, which is generally between 1 to 8 weeks. In humans, the incubation period can reach 6 months to several years.[7,14,21,23,27] Viral presence in the saliva occurs only after passing through and replicating in the central nervous system, and virions are intermittently seen in some, but not all, salivary glands.[24] Therefore, the diagnosis of rabies in mammals can only be achieved with 100% certainty through the post-mortem examination of the central

nervous system or trigeminal ganglion.[7,20,21] The most significant aspect of rabies diagnosis is rapid and accurate detection in animals suspected of exposing a human to the virus, so that proper post-exposure treatment decisions can be made.

Despite the serious clinical outcome of rabies virus infection, histopathological changes seen in the central nervous system are often just characteristic of viral encephalitis. Inflammatory lesions can be very mild or even absent.[14,18,21] Because the encephalitis induced by rabies can be hard to distinguish from other viral diseases that affect the CNS, rabies should always be included in a differential diagnosis when neurological signs and encephalitis are present.[3,7,17,18,20,21,24,27] For the most part, inflammatory changes are characterized primarily by perivascular cuffs (Figure 1), variably-sized aggregates of glial cells in the parenchyma (gliosis), and intracytoplasmic inclusion bodies may also be found.[7,18,24-27]

For over a century, the diagnosis of rabies was based on the histologic observation of hematoxylin and eosin (H&E) sections for round, acidophilic inclusions in the cytoplasm of neurons, named Negri bodies after their discoverer, an Italian pathologist, Adelchi Negri (Figure 2).[2,11,25] Although the presence of Negri bodies are pathognomonic for rabies, many studies revealed they are present in only 50-80% of rabies cases.[1,11,16,21,25,27] Rarely, nonspecific protein inclusion bodies are found in certain areas of the brain in different species, which can be easily misinterpreted as rabies virus inclusions by inexperienced pathologists, generating false-positive diagnoses when relying on this method alone.[15,16,21] In the late 1950s, histological tests began to be superseded by more sensitive immunological tests that could specifically detect rabies virus antigen.[2] The direct fluorescent antibody test (FAT) was developed, and is still widely used as the standard in rabies diagnosis today.[11,18,21,25] Despite its efficiency in quickly detecting rabies, FAT has many drawbacks that can limit its usefulness. This technique is costly since it requires

the use of expensive ultraviolet light microscopes. The impermanent fluorochrome dyes used in the protocol fade very quickly, and tissues stained with fluoresceinated antibodies cannot be counterstained for other histological examinations of tissues.[1,11] Though FAT testing remains the preferred method for routine rabies diagnosis in fresh brain material, these tissues contain live viruses and constitute a biosafety hazard for handlers.

Over the last 20 years, immunohistochemistry (IHC) has become increasingly popular for detecting numerous antigens in fixed tissues using light microscopy.[11,21,27] The IHC staining techniques are widely used on formalin-fixed paraffin-embedded tissues in human and veterinary medicine for the diagnosis of bacterial, viral, parasitic and fungal diseases.[1] General IHC staining procedures exploit antigen-antibody affinity, visually marking the antigen with primary antibodies and conjugated secondary antibodies. At the end of the reaction, the antigen appears as fine granules or as homogeneous red or brown discoloration within the target cell, depending on the chosen chromogen (Fast Red or DAB).[26] Numerous studies have shown the sensitivity of IHC for rabies is equal to that of FAT, and it has been reported that IHC is even more sensitive in establishing an early diagnosis of suspected cases when traditional histological and FAT techniques could not detect viral antigens or lesions.[1,12,27]

Immunohistochemistry was used in this study to analyze the distribution of rabies virus antigen in representative parts of brain of several species. Since rabies does not always infect all segments of the brain equally and the spread differs from species to species, such varying degree of antigen presence can compromise the reliability of test results if all parts are not sampled, or if a sample has low levels of viral antigen. The collection of dependable samples is therefore essential for optimizing diagnosis.[2] Those involved in rabies surveillance, collection and testing will benefit from knowledge of the primary brain parts to use for detecting viral antigen, and it is

crucial to establish these preferred sites in instances in which composite evaluation is not available. Immunohistochemistry may have very practical applications in diagnostic centers in developing countries where FAT testing is difficult, sampling of the brain occurs in the field, and rabies remains a major health risk for humans.

The main objectives of this study were to develop an immunohistochemistry protocol with a polyclonal antibody suitable for diagnosis in a variety of mammalian species, examine the reliability of that technique, and develop a preliminary idea about geographic regions of the brain potentially useful for making future diagnoses in various species.

CHAPTER 2
MATERIALS AND METHODS

Cases

All formalin-fixed paraffin-embedded (FFPE) samples were obtained from the Department of Pathology, Athens Diagnostic Laboratory, and Southeastern Cooperative Wildlife Disease Study at the College of Veterinary Medicine, University of Georgia, Athens. These cases had been previously diagnosed with rabies by FAT and/or histopathological examination. Immunohistochemistry was performed on a total of 37 cases, encompassing 13 different mammalian species. These rabies cases included: 7 skunks (*Mephitis mephitis*), 7 raccoons (*Procyon lotor*), 5 bovine, 4 cats, 3 dogs, 3 horses,1 swine, 1 llama, 2 gray foxes (*Urocyon cinereoargenteus*), 1 red fox (*Vulpes vulpes*), 1 bat (*Myotis sp.*), 1 white-tailed deer (*Odocoileus virginianus*), and 1 bobcat (*Lynx rufus*).

Immunohistochemistry Staining

All tissues were examined by immunohistochemistry using the following protocol to detect viral ribonucleoprotein for *Lyssavirus*. After deparaffinization in CitriSolv, tissue sections were quenched with 3% hydrogen peroxide and rinsed in water. The antigenic sites were exposed by microwaving in a citrate buffer solution (1X Vector Ag-Retrieval Unmasking Solution) for 10 minutes (4'-4'-3' intervals). A 1:10 diluted Powerblock solution (Biogenex Universal Blocking Reagent) was applied to each slide for 7 minutes. The tissues were incubated at room temperature for 1 hour with 500µl of rabies polyclonal primary antibody (made in goat), diluted to 1:2000. This was followed by a 20-minute incubation in biotinylated link antibody (LSAB2 Kit) at room temperature and another 20-minute incubation in HRP conjugated

streptavidin (LSAB2 Kit). The reaction was revealed in DAB peroxidase substrate (Dako Laboratories) for no longer than 5 minutes. The slides were counterstained with Mayer's hematoxylin and coverslipped using permanent mounting medium (Dako). All positive control sections were from an FAT-positive rabies case demonstrating inclusion bodies. Negative control sections, which were from rabies-positive brains as well, were treated with 500µl PBST instead of primary antibody.

Viral Antigen Distribution

For each species, at least four different anatomic locations in the brain were examined, including: brainstem, cerebellum, cerebrum (including the cerebral cortex and subcortical white matter), and hippocampus. The samples of the horses also included cranial cervical spinal cord and were tested as well. The IHC staining results were evaluated for the degree of rabies antigen intensity. The positive signal intensity in each section was scored as follows: no staining present (-); rare foci of positive signal with an average of one per high power field (+); 2-5 foci of positive signal on average per high power field (++); and extensive positive staining with greater than 5 cells per high power field (+++). Distribution scores were used to designate the best section of brain to apply IHC only in those species for which there were more than 3 cases available. The remaining species' results were used to evaluate IHC accuracy and applicability, and to possibly give insight into other distributional differences of antigen.

CHAPTER 3
RESULTS

Results of immunohistochemistry for rabies antigen in the brain sections of various species are presented in Table 1. This immunohistochemistry protocol successfully detected rabies virus antigen in all 37 cases. The signal was observed almost exclusively in the gray matter of the different areas, characterized by sparse to diffuse granularity or variably-sized brown dots throughout the perikaryon of the different neurons and their axonal and dendritic processes (Figures 3-15). No staining was observed in the white matter. Some cases demonstrated inclusions as brown oval homogeneous structures within the perikaryon of neurons (Figure 3). The presence of the viral antigen in the processes of the neurons gave a granular appearance to the neuropil, and was sometimes found trailing through variable lengths of axons (Figure 4).

The application of this technique to main segments of the central nervous system highlighted distributional differences of rabies antigen for each species (Table 1). For the domestic carnivores, i.e., cats and dogs, results were very similar. In these species, the hippocampus had the most intense signal scores, followed by the cerebrum, brainstem, and cerebellum, respectively (Figures 5-6).

In the bovine cases, the most striking signaling was seen in the perikaryon of large neurons of different nuclei in the brainstem. In most cases, the entire cytoplasm stained as dark brown and antigen was more diffuse. The cerebellum of the bovine cases showed the second highest viral intensity, and Negri bodies in the Purkinje cells were observed as highly stained, oval homogenous structures (Figure 3). Throughout the molecular layer of the cerebellum,

dendritic processes were evident with strong labeling. There was patchy labeling in the cytoplasm of the granular cell layer with a strong signal seen in the Golgi neurons.

For horses, the cervical spinal cord obtained for each case showed the most intense labeling, followed by the brainstem. In all three horses the distribution of the viral antigen was regularly diffuse throughout the neuraxis (Figure 7), with sparing or even absent distribution in sections of the hippocampus, cerebellum and cerebrum. Antigen granularity in horse brain and spinal cord, although consistently diffuse, was much less dense and infrequent. Comparing the horse cases with other species, especially bovine, the labeling within the perikaryon of neurons was more distinct and sparse.

In raccoons (*Procyon lotor*) and skunks (*Mephitis mephitis*), viral intensity varied between moderate and severe in all examined segments; however, in all cases the hippocampus was the site where the signal was consistently severe (Figures 8-9). IHC staining of any of the brain segments yielded positive results.

IHC was only applied to one swine case, and the results revealed moderate reactivity in the brainstem, with milder reactions in the other segments (Figure 10). The one llama case studied had intense staining in all segments (Figure 11).

Both red and gray fox species (*Vulpes vulpes, Urocyon cinereoargenteus*) demonstrated the most viral antigen staining in the cerebral cortex, followed by the brainstem (Figures 4 and 12). In the remaining wildlife species studied with IHC, positive staining was detected in each case for the areas of the brain that were available (Figures 13-15).

Table 1. Degree of rabies antigen intensity in different sections of the brain in 13 mammalian species[a]

Species (No. of cases)	Brainstem	Cerebellum	Hippocampus	Cerebrum
Raccoon (7) *(Procyon lotor)*	++	+	+++	+++
	++	N/A	+++	+++
	++	+	+++	+++
	++	++	+++	+++
	N/A	N/A	+++	+++
	++	++	+++	+++
	++	+	+++	++
Skunk (7) *(Mephitis mephitis)*	++	N/A	+++	++
	+	+	++	++
	++	++	+	++
	+++	++	+++	++
	++	++	++	++
	N/A	+	N/A	++
	+++	+++	+++	++
Bovine (5)	+++	N/A	+	+
	+++	N/A	-	-
	+++	++	-	++
	++	+++	-	++
	++	+	-	+
Cat (4)	++	+	+++	+++
	++	++	+++	+++
	++	++	+++	+++
	+	+	++	+
Dog (3)	++	++	+++	++
	+	-	+	-
	++	+	++	++
Horse (3)[b]	+++	+	++	+
	+++	++	N/A	++
	++	+	-	+
Llama (1)	+++	+++	+++	++
Swine (1)	++	+	+	+
Red fox (1) (*Vulpes vulpes*)	+	-	N/A	++
Gray fox (2) *(Urocyon cinereoargenteus)*	++	-	+	++
	+	+	+	+
White-tailed deer (1) *(Odocoileus virginianus)*	++	N/A	N/A	++
Bobcat (1) (*Lynx rufus*)	+++	+++	+++	+++
Bat (1) (*Myotis* sp.)	+++	++	+	+

[a] *Scores were based on the following scale: absent signal (-), mild signal (+), moderate signal (++), severe signal (+++), or segment of brain was not obtained for that case (N/A)*

[b] *The cervical spinal cord was also obtained and analyzed for each horse. The signal intensities were: (+++), (+++), and (++)*

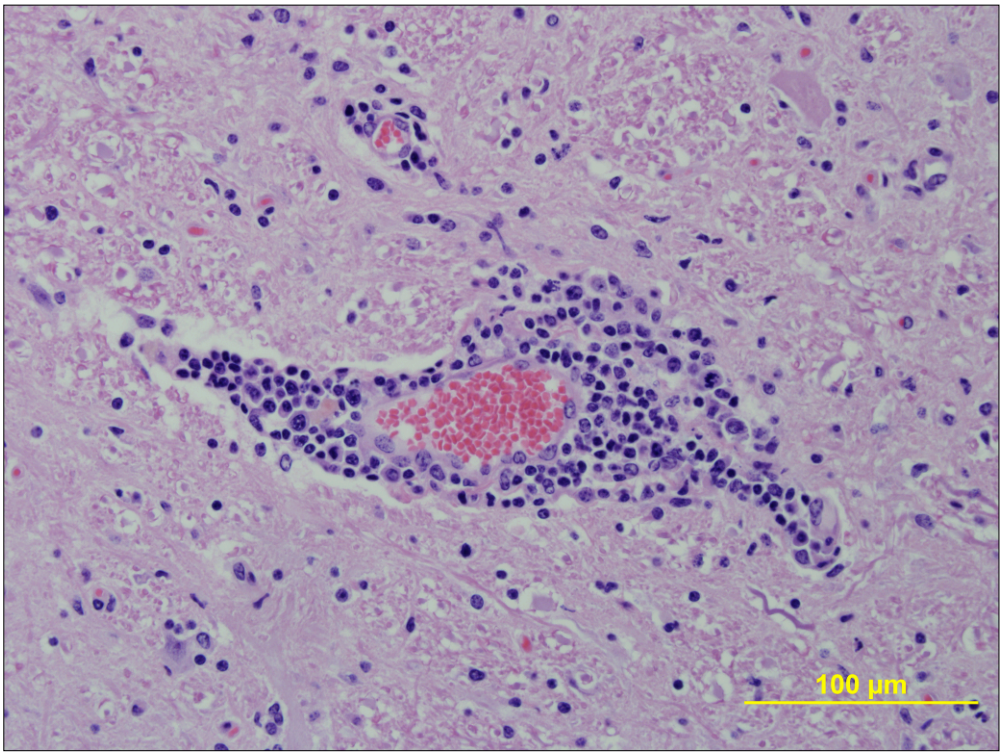

Figure 1. Bovine. The space of Virchow-Robin (perivascular space) is expanded by two to three layers of inflammatory infiltrate composed of lymphocytes and plasma cells. H&E stain.

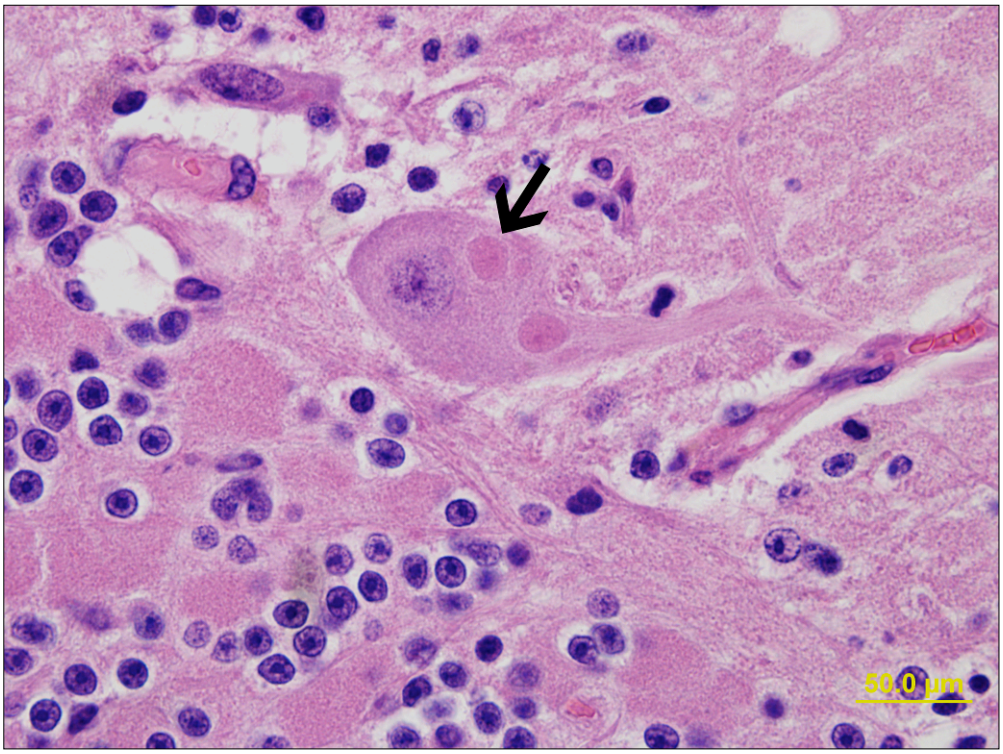

Figure 2. Bovine. The perikaryon of a Purkinje cell of the cerebellum contains two opaque, oval eosinophilic inclusion bodies (arrow). These inclusions are called Negri bodies and are characteristic of rabies infection. H&E stain.

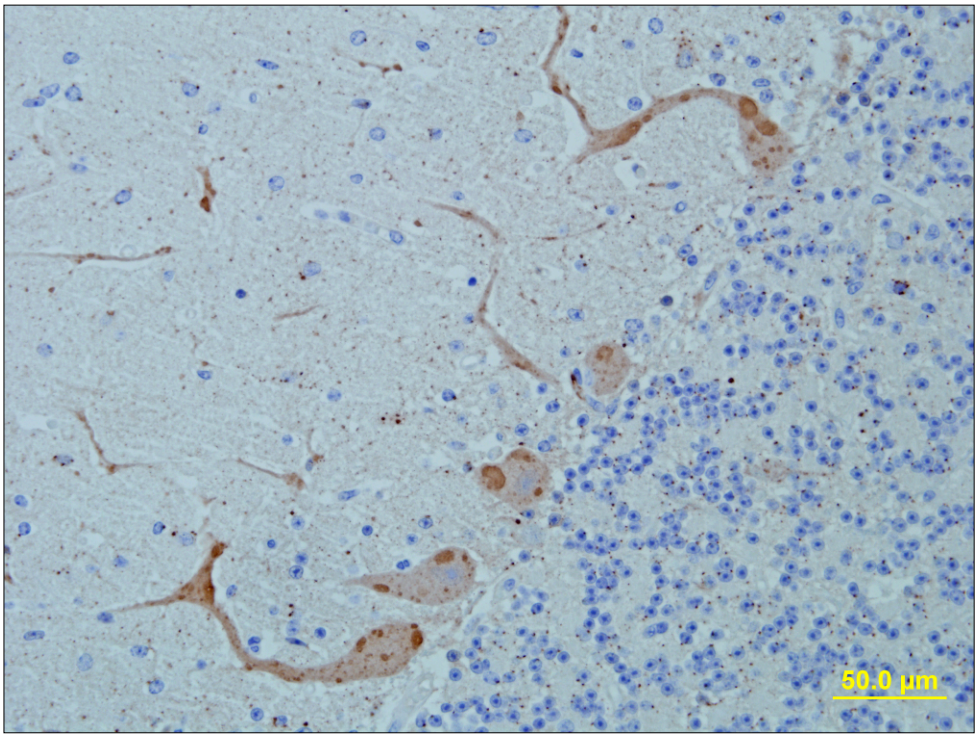

Figure 3. Bovine. Several brown, circular, strongly positive inclusion bodies are seen within the cytoplasm of Purkinje cells in the cerebellum. The dendrites of many Purkinje cells are also strongly positive. The granular cell layer shows widespread fine granularity. LSAB method with DAB substrate. Mayer's hematoxylin counterstain.

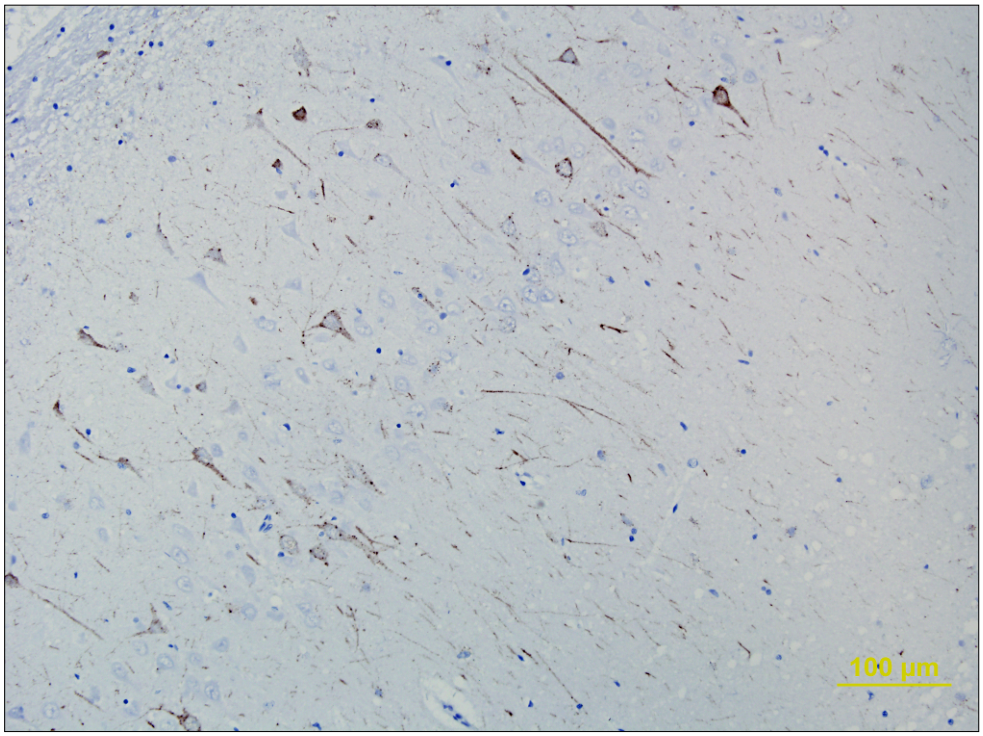

Figure 4. Red fox (*Vulpes vulpes*). Positive brown granularity within the cytoplasm and processes (axons and dendrites) of neurons in the hippocampus. LSAB method with DAB substrate. Mayer's hematoxylin counterstain.

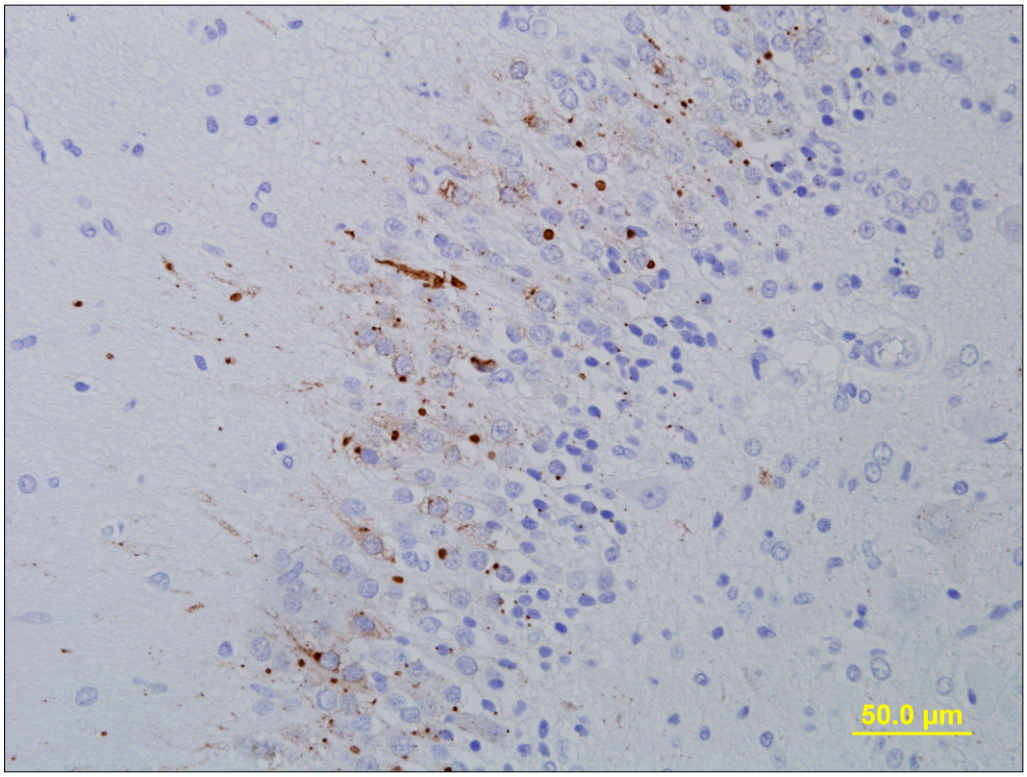

Figure 5. Dog. Distinct brown granularity in the cytoplasm of several neurons in the hippocampus. LSAB method with DAB substrate. Mayer's hematoxylin counterstain.

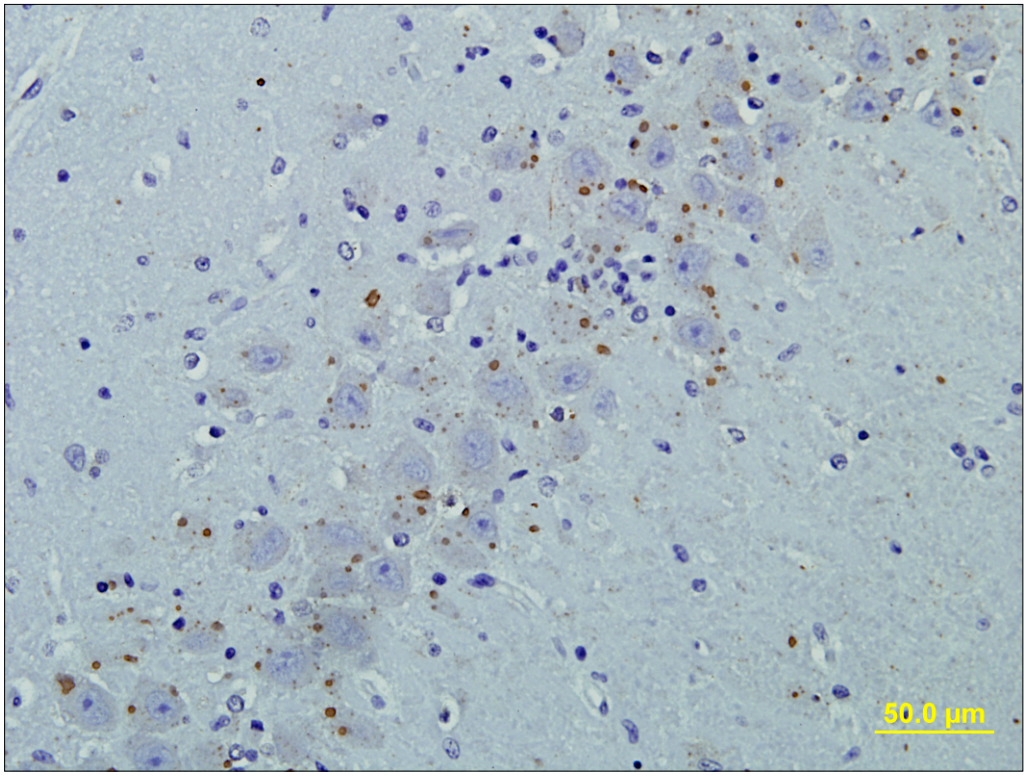

Figure 6. Cat. Multiple neurons of the hippocampus showing several brown dots within their perikaryon. LSAB method with DAB substrate. Mayer's hematoxylin counterstain.

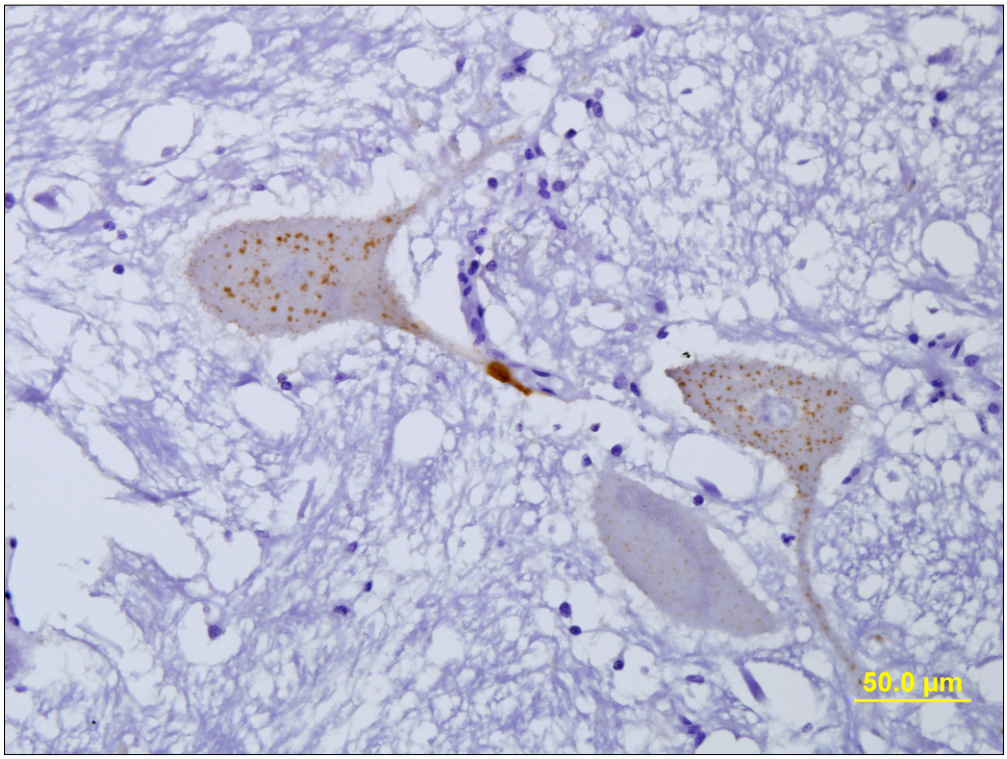

Figure 7. Horse. Small brown granules of viral antigen within the neurons and axons of the pons. LSAB method with DAB substrate. Mayer's hematoxylin counterstain.

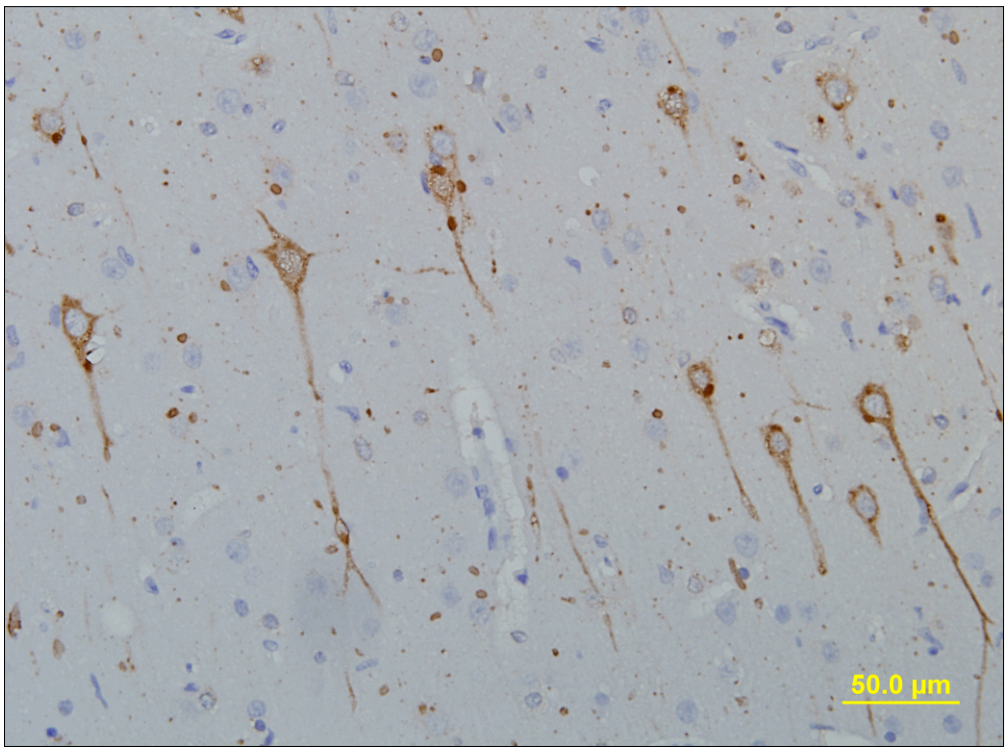

Figure 8. Raccoon (*Procyon lotor*). Positive brown granularity and inclusion bodies are highlighted within the cytoplasm of hippocampal neurons. Viral antigen is also seen tracing through neuronal processes. LSAB method with DAB substrate. Mayer's hematoxylin counterstain.

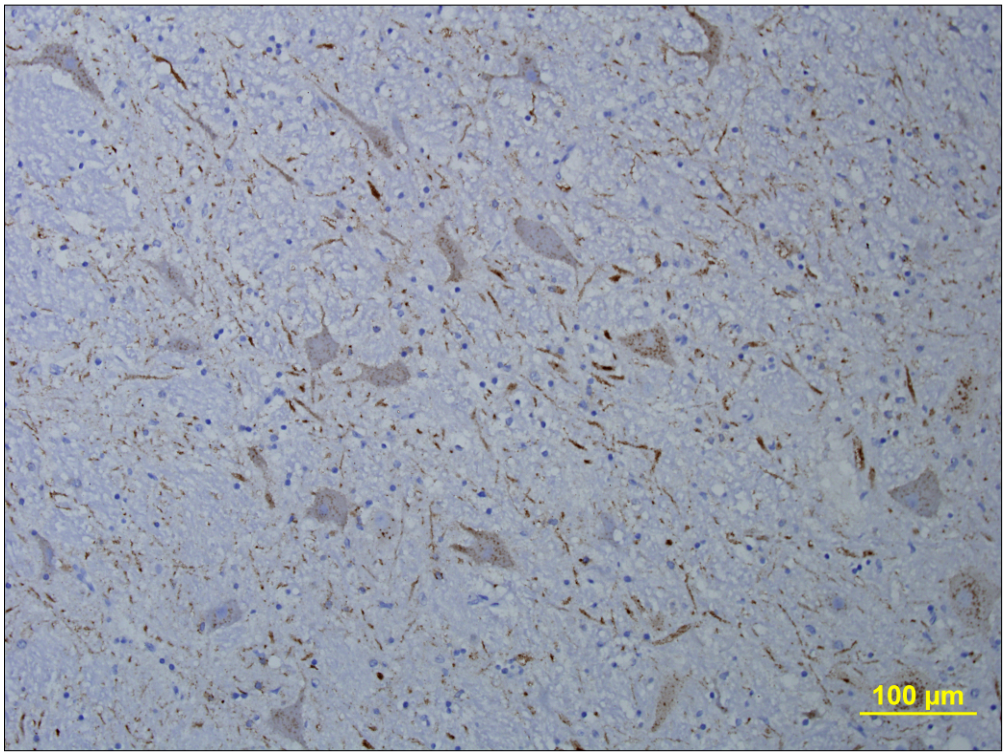

Figure 9. Skunk (*Mephitis mephitis).* Brown viral antigen dispersed widely throughout neuronal perikaryon and their processes in a nucleus of the brainstem. LSAB method with DAB substrate. Mayer's hematoxylin counterstain.

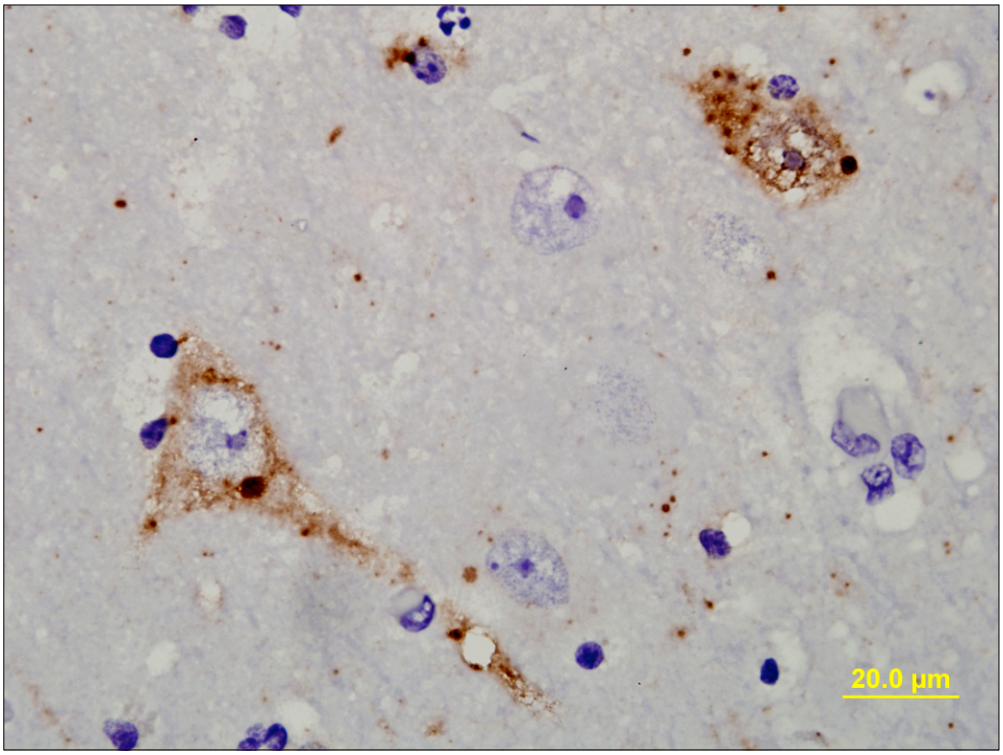

Figure 10. Pig. Neurons of the cerebral cortex demonstrate small clumps of viral antigen and well-defined circular spots. LSAB method with DAB substrate. Mayer's hematoxylin counterstain.

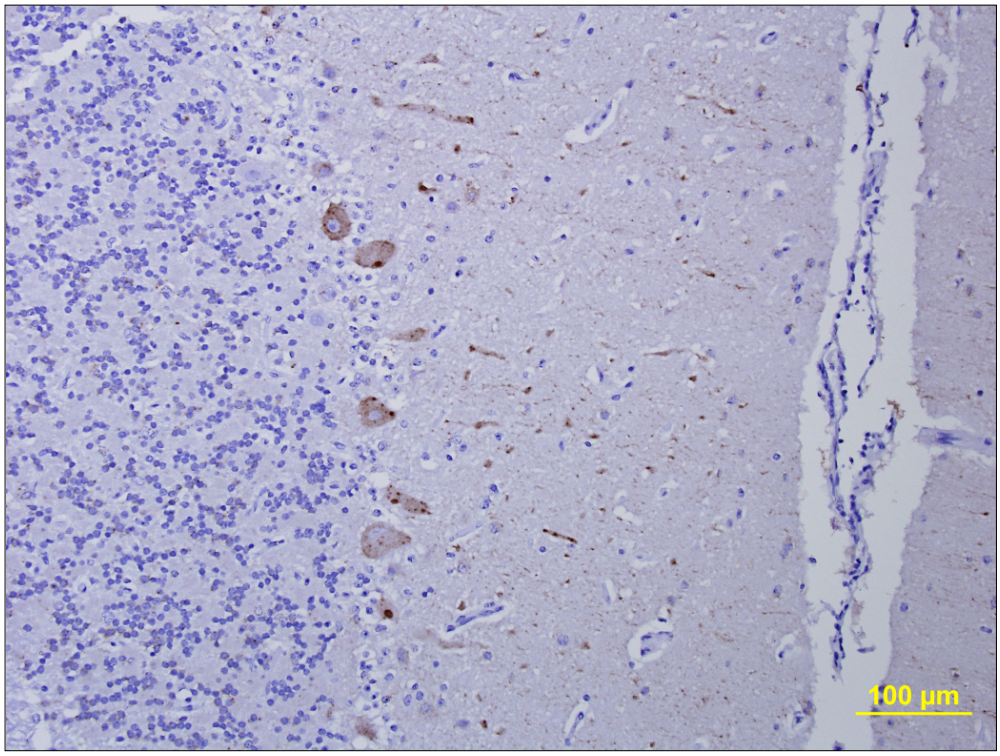

Figure 11. Llama. Viral antigen is observed in the cytoplasm of the Purkinje cells and their dendrites extending into the molecular layer of the cerebellum. LSAB method with DAB substrate. Mayer's hematoxylin counterstain.

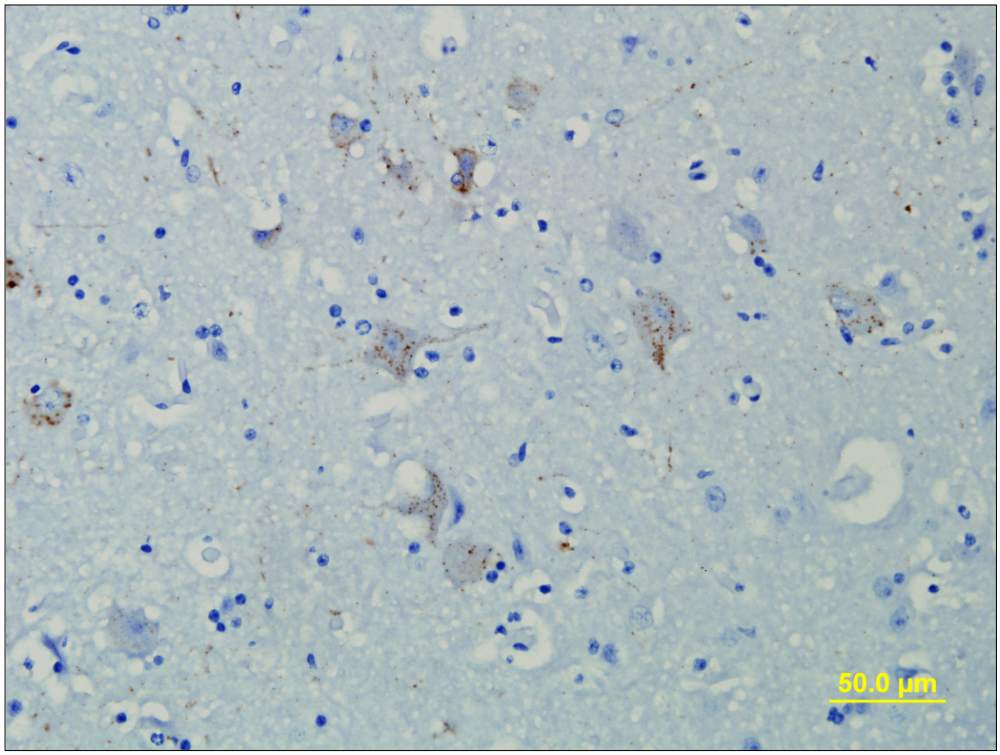

Figure 12. Gray fox (*Urocyon cinereoargenteus*). Viral antigen stained positively in multiple neurons of the brainstem. LSAB method with DAB substrate. Mayer's hematoxylin counterstain.

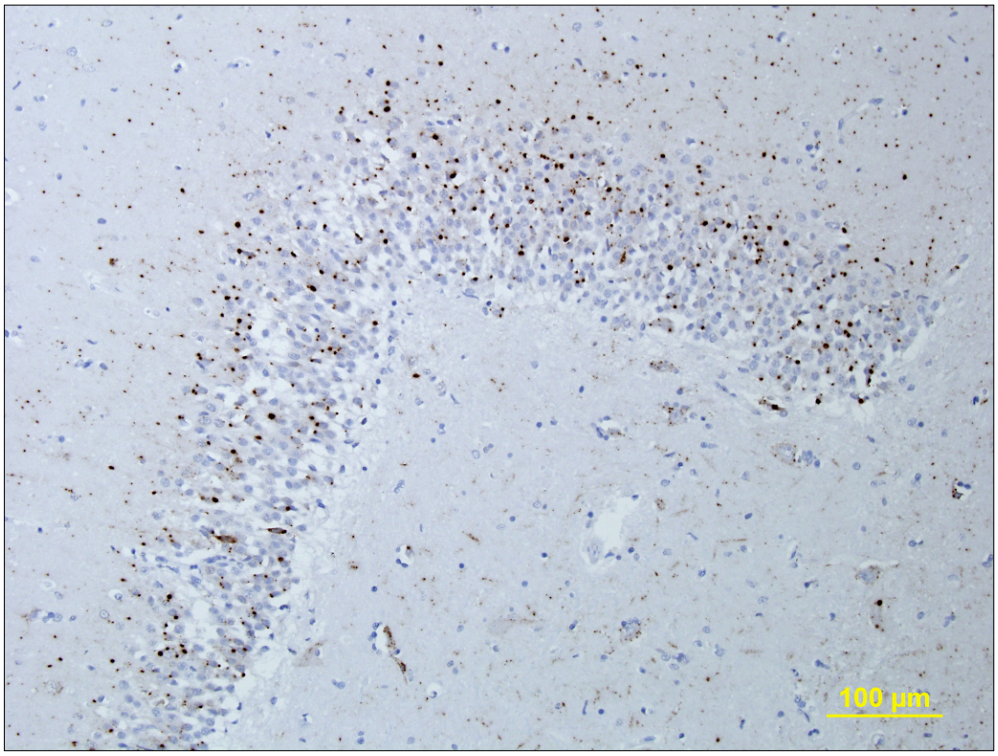

Figure 13. Bobcat (*Lynx rufus*). The cytoplasm of neurons throughout the hippocampal formation show multiple fine brown granules of viral antigen. LSAB method with DAB substrate. Mayer's hematoxylin counterstain.

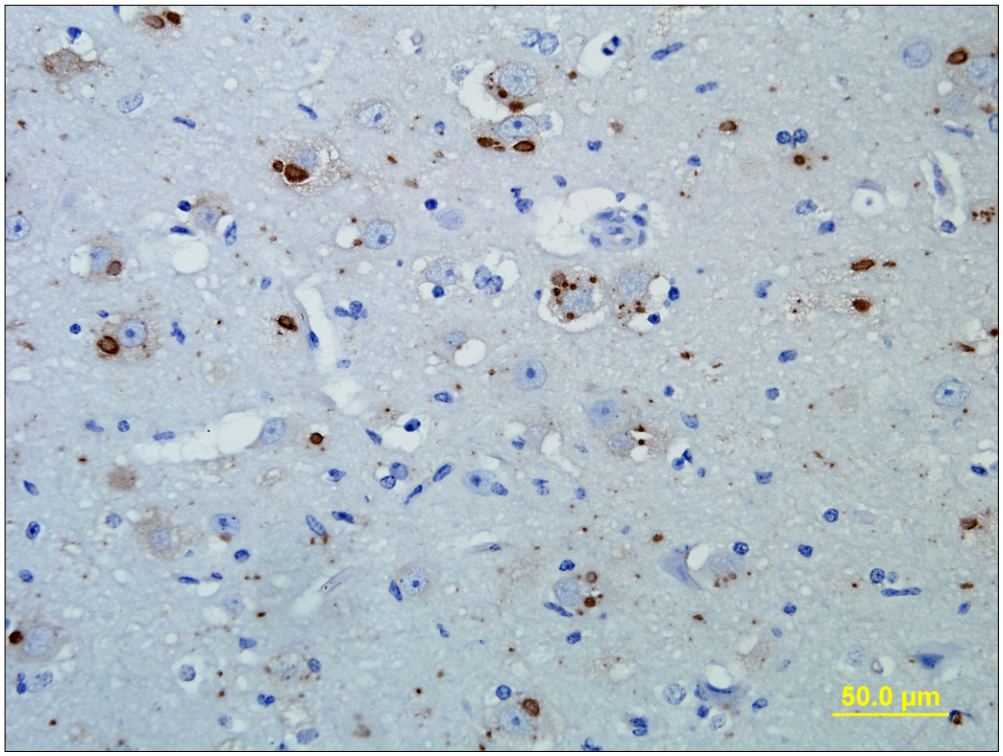

Figure 14. White-tailed deer (*Odocoileus virginianus*). Intracytoplasmic inclusion bodies and fine antigen particles found within several neurons of the cerebral cortex. LSAB method with DAB substrate. Mayer's hematoxylin counterstain.

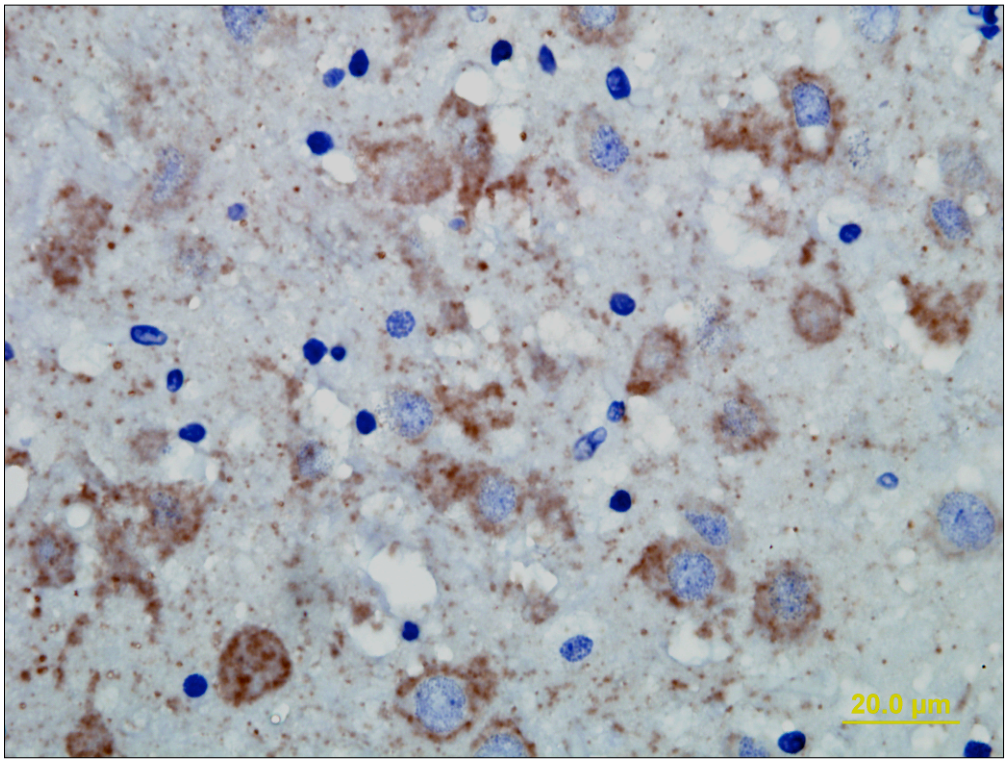

Figure 15. Bat (*Myotis* sp.). Strong signaling diffused widely throughout the cytoplasm of the neurons in the brainstem. LSAB method with DAB substrate. Mayer's hematoxylin counterstain.

CHAPTER 4
DISCUSSION

Rabies was confirmed in all 37 cases using immunohistochemistry, showing this method is 100% sensitive. The viral antigen distributed throughout the brain was consistently and accurately detected in all mammalian species. Since the polyclonal antibody used in this technique, which is commercially available, was also used on some cases from Brazil, it has potential as a universal test.

Historically, certain parts of the brain were considered reliable places for detecting Negri bodies, or were sent for FAT to confirm the diagnosis of rabies. These parts included the hippocampus in carnivores and the cerebellum in herbivores.[2,11,15,18,27] The initial diagnostic focus on hippocampus sections can be attributed to their high frequency of large antigen aggregates, which are easily identified by traditional H&E stains.[11] Decades of histological searches for Negri bodies in rabies cases led to an understanding that numerous factors cause uneven and inconsistent distribution of these inclusions. The site of exposure, host susceptibility and age, strain of virus, type of virus form (whether paralytic or furious), amount of inflammation, length of clinical infection, and cause of death (perhaps premature with euthanasia) could all contribute to differences in size and frequency of Negri bodies.[2,3,6,7,18,23,25] The accuracy of laboratory diagnosis was greatly reduced when relying solely on this histological finding, which was only being found in about two-thirds of rabies cases, or less, in many species.

Along with such variable and inconsistent Negri body formation, some inclusions seen in H&E staining in the cytoplasm of neurons resemble Negri bodies, but might not actually be

rabies-specific. Such pseudo-Negri bodies are protein-related inclusions and could lead to an initial false-positive diagnosis of rabies, since inflammatory changes are not always found in all rabid cases. In a study of 8 nonrabid dogs, inclusion bodies that were morphologically indistinguishable from Negri bodies were found in the thalamus, Purkinje cells, and cerebral cortex.[15] Similar intracytoplasmic inclusion bodies have been noticed in neurons of nonrabid cats, skunks, cattle, moose and woodchucks as well.[14,15,23,25] For these reasons, it became clear that rabies diagnosis could not simply rely on the presence of Negri bodies in the hippocampus, but that IHC or FAT should be applied to rabies-suspect cases in order to confirm finer distributions of viral antigen.

This study has shown there are distinct distributional differences of rabies virus antigen in the brain, therefore selection of samples for testing is important. Numerous studies have analyzed the spread of rabies viral antigen in the brain using other diagnostic measures more sensitive than H&E staining. A comprehensive study of multiple species using FAT concluded that when composite sampling of the brain is not possible, the brainstem, preferably the thalamus, should be the area of choice for testing.[2] Meanwhile, other histological studies deemed the medulla, hypothalamus and tegmental region of lower brainstem as the most commonly infected sites in rabies.[7,20] Despite this range of results, authorities continued to recommend that the hippocampus and cerebellum remain the two sites for preferred rabies testing. Most sampling recommendations are based on obsolete histological tests or FAT analysis, and few studies have assessed the reliability of individual segments specifically for IHC.[2] Past studies have identified reliable brain areas for generalized rabies diagnosis, but did not address viral distribution with respect to individual species. Our results are the first to give more insight into IHC testing preference relative to species variation.

Dogs and cats were the two domestic carnivores studied, and revealed very similar results with respect to brain part predilection of the virus. Using IHC to evaluate four different segments of brain, the hippocampus will yield the most reliable results in future suspect cases of these species. Though rabies in dogs and cats has been greatly reduced in the United States, unvaccinated individuals may contract the virus from wildlife reservoir species and expose humans to rabies that they are in close contact with. Despite widely held associations of rabies with dogs, it should be noted that there are more reported cases of rabid cats in the US than any other domestic animal, with nearly 200-300 cases annually.[7,10] Canine rabies remains of the highest importance in developing countries where it is estimated that nearly 48% of dogs are rabid and constitute the majority of human transmissions and 90% of human deaths worldwide.[7,22] Confining and observing a suspect cat or dog involved in a human bite may not be possible or even reasonable, especially where rabies is uncontrolled. Diagnosis in these species must be extremely quick and accurate to make proper human post-exposure treatment decisions.

After assessing distributional trends of antigen with IHC in five cattle, the brainstem proved to be the area of the brain with most signaling. Any attempts at proper diagnosis should include this region and the cerebellum, which also showed moderate to strong signaling. These herbivorous species, though they act as dead-end hosts for viral transmission, are commonly infected through bites by the typical reservoir species in their geographic area. The greatest economic and public health impact of rabies in cattle exists in Latin America, where the paralytic form of the disease is commonly transmitted by vampire bats.[7,17] In many countries, spillover infection to livestock and horses often appears to coincide with wildlife epizootics.[5] In Brazil, farm animal losses caused by rabies are very considerable and it is estimated that over 842,000 cattle deaths are caused by rabies each year in this country.[3,13,23] Rabies in herbivorous species

such as cattle can show great variance in not only the clinical signs, but also the distribution of lesions. Though Negri bodies in cattle and other small ruminants are found in about 85% of cases, there is still the need for IHC to identify rabies antigen in any case where these inclusions are not present.[13]

In this study, horses showed the highest intensity of antigen in the cervical spinal cord, followed by the brainstem, with consistent positive signaling throughout the neuraxis and sparing or even absent distribution in sections of the hippocampus, cerebellum and cerebrum. In horses, there has been much variation in the distribution of rabies antigen in several previous studies using Negri body mapping or FAT, leading to an inconsistency in the accurate place being used for FAT diagnoses. Horses usually develop a much lower number of Negri bodies than any other species, sometimes in only 30% of cases,[5,13,17,26] perhaps paralleling their inconsistent and minimal viral presence. Most studies assessing horse rabies have incorporated FAT, which has shown to be less sensitive for equine samples when compared to other animal species.[3] In a FAT study of equine rabies in 4 regions of the brain, only 29% of samples were positive for all 4 regions,[3] also confirming this highly selective and limited viral distribution. Many diagnosticians and researchers claim the cerebellum provides the best tissues for FAT simply based on bovine rabies, which could also be causing misdagnoses.[3] Despite a high discrepancy in reports of viral distribution in horse cases, the spinal cord is a reliable location for the detection of rabies antigen based on these IHC results. Since inclusion bodies are seldom encountered and viral antigen is sparse, the sensitivity of IHC in rabid horses may become very beneficial to confirm the diagnosis.

In 2003, 63 rabies cases were reported in horses and mules in the United States, with an 8.6% increase from the previous year.[26] Equine rabies is often considered a silent disease

because clinical signs commonly go unnoticed, but diagnosis in this species is becoming more critical as their popularity in recreation and exposure to humans increases.[3] A single positive diagnosis of rabies in one pony in 2003 on a visiting farm in Connecticut brought 67 people forward after having contact with the pony in the two weeks prior to its diagnosis.[3] Even though the transmission of virus from horses to humans is rare, there is still a potential risk for veterinarians and horse owners, and human risk to rabies in central and south America is becoming more significant.[5] The number of positive cases in São Paulo, Brazil has grown, with more than 570 equine cases between 2000 and 2003. These numbers are of high concern because horses are being used extensively in recreational activities with frequent handling by children and adults, and regions such as São Paulo are placing a big diagnostic emphasis on identifying the brain regions which accumulate the most antigen.[3]

Rabies is also now considered the most notable naturally occurring viral infection in New World camelids such as llamas and alpacas. Their increasing popularity in North America and propensity to bite has forced rabies to be included in the differential diagnosis when acute encephalitis is suspected. With four positive cases in llamas between 2002 and 2004 in the US, surveillance in these mammals must be monitored through proper diagnosis, which IHC proved to do in this study.[7]

Looking outside of the main domestic species used in this study, the wild species samples were also confirmed to have rabies using IHC, which gives insight into the most widespread and complex realm of rabies virus. The application of IHC to the brain selections of raccoon (*Procyon lotor)* and skunk (*Mephitis mephitis*) pointed out the hippocampus as the most prevalent source of antigen, but any sample from the brain in these species could be used for a proper diagnosis. In an FAT study of rabies antigen distribution in different brain structures, wild

carnivores (mostly mongooses [*Cynictis penicillata*], jackals [*C. mesomelas*] and bat-eared foxes [*Otocyon megalotis*]) contained a higher frequency of antigen throughout all brain regions when compared to domestic dogs and cattle.[2] Only one wild animal brain in their study had localized antigen, showing that rabies virus might be less confined in certain wild animal cases and sampling could be less strict.[2] Serving as two of the major rabies virus reservoirs in North America, the prominent presence of antigen throughout the brains of raccoons and skunks corresponds with the rabies cycle, in which these animals are responsible for most transmissions of the disease. High virus levels might enhance transmission through the saliva, therefore leading to increased viral replication in the central nervous systems of wild carnivores. Red and gray foxes (*Vulpes vulpes, Urocyon cinereoargenteus)* showed more viral presence in the cerebral cortex and the brainstem and also constitute a large reservoir population globally. The single bat case analyzed (*Myotis* sp.) also demonstrated positive reactivity.

Sylvatic (wildlife) rabies is still enzootic in the US with 7,000 to 8,000 wildlife rabies cases being reported every year.[26] These species have contributed almost all of the transmissions to humans in the United States over the past few decades.[14] Oral vaccinations attempts in this immense wild population through vaccine-laden baits have shown some success, but must involve more development and other considerations specific to reservoir populations in each area before massive implementation.[7,20,21,23,24]

Raccoons, skunks, foxes and bats alone accounted for 91% of rabies cases in 2000,[9] and the diagnosis in these wild reservoir species is of utmost importance, since their populations are extensive and spillover into livestock, domestic animals and humans is prominent. Foxes are one of the most widely distributed and abundant wild carnivores in the world, raccoons are the sole major terrestrial carnivore in the US, and skunks are the second most recorded species amongst

US wildlife rabies enzootics.[7] Foxes and raccoons are highly susceptible to small inoculations of virus, and the host-virus relationship in each of these species facilitates the maximum amount of viral shedding in the saliva and appropriate behavioral alterations to allow transmission via bites. Skunks in the United States, though their infections are associated with raccoon rabies virus variants, show a likelihood of soon becoming their own independent reservoir.[7]

Bats, which are reservoirs of rabies on all inhabited continents, host 6 of the 7 identified lyssavirus genotypes and are the major source of human rabies in New World, western Europe and Australia.[7] They also contributed to 39 human cases in the last 50 years in the US. The great diversity of bat species throughout the world makes their contribution to rabies enzootics vast, and also independent from terrestrial rabies transmission cycles.[20] Rabies is more commonly found in hematophagous vampire bats, as opposed to bats of the *Myotis* genus, such as the one in this study. *Myotis* bats, though they are insectivorous, can still transmit rabies, just not nearly as likely. Monoclonal antibody and phylogenic testing has revealed more than 30 different lineages of rabies virus found in all bats, and a standard test such as IHC that can identify all of these variants would be extremely beneficial.[24]

While the majority of wildlife cases in the United States occur in these four species, between 1960 and 2000 there were a total of 2,851 cases of rabies in 17 other carnivore species reported to the CDC.[7,9] These other species included mongooses (*Herpestes javanicus*), coyotes (*Canis latrans*) and bobcats (*Lynx rufus*), and the rabies virus variants detected corresponded with variants of the major terrestrial reservoirs.[7,9] The numbers of these other carnivorous mammals with rabies doubled between 1990 and 2000, corresponding to several epizootics of rabies among coyotes in southern Texas, a 5-fold increase in the number of rabid bobcats, and a 300% increase in rabid mongooses in the past few decades.[9] Rabies in carnivores outside of the

primary grouping of reservoir host species is not that common in the United States, but must still be considered in surveillance and the protection of humans and domestic animals from exposure.[9,21]

There are multitudes of other species that have been infected with rabies, though in very low frequencies, and persist as spill-over victims of the main virus variants in North America. These species include: wolves (*Canis lupus*), badgers and otters (Mustelidae family), ringtails (*Bassariscus astutus*), beavers (*Castor* sp.*)*, woodchucks (*Marmota monax*), squirrels (Sciuridae family), guinea pigs (*Cavia porcellus*), domestic rabbits (*Oryctolagus cuniculus*) and domestic ferrets (*Mustela putorius*), all of which could possibly transmit the virus to humans.[4,7,9,10] There is a wide diversity of rodent species that come into contact with common reservoirs and have shown competence as hosts for other viral diseases.[7] From 1985 to 2004, a surprising 33 rabid beavers were recorded in the eastern United States, all within a corresponding raccoon rabies enzootic.[7] Very little is known about rabies pathogenesis in beavers and many other wild rodents, and it can be often difficult to discern aberrant behavior in these species. A wounded otter in Georgia confirmed to have rabies led to a multistate search for exposed persons who would need to receive PEP treatment.[9] In New York in 2003, an unsuspected rabid guinea pig bit its owner, which eventually led to PEP administration, and in the same area between 1992 and 2001, 7 domestic rabbits were confirmed to have rabies.[4] Rabies vaccines are only approved for dogs, cats and recently for ferrets, and pet guinea pigs and rabbits are highly susceptible to rabies infection, considering Pasteur used rabbits for the creation of the first rabies vaccine.[10,20,24] Since clinical manifestations in many species are very unpredictable and hard to detect, it is imperative that the best rabies diagnostic tool be able to identify rabies in any mammalian species in such an

uncontrolled wildlife domain. Based on the ubiquitous success of this IHC protocol, it could be utilized in spillover instances and in rabies cases outside of the primary host reservoirs.

Immunohistochemical studies of the distribution of rabies virus antigen can be administered with polyclonal or monoclonal anti-ribonucleoprotein antibodies. However, polyclonal antibodies can detect multiple epitopes of rabies virus and staining is noted to be more sharp with polyclonal antibodies than monoclonal.[8] Some IHC studies only use monoclonal antibody preparations, which may not be suitable especially if their activity depends on conformational epitopes.[20] This particular polyclonal antibody successfully detected rabies virus in all 13 species, despite possibly antigenically-differing viral strains.

It is known that the population of rabies virus contains genotypically and phenotypically distinguishable virus variants that are adapted to and maintained by a few mammalian species.[7] Any given viral lineage is always associated with a certain reservoir host, in which the unique host-virus relationship drives animal behavior to enhance virus transmission by maximizing viral shedding in the saliva. Isolates from one host often have a different pathogenicity for other species, suggesting a degree of viral selection as well.[7,23] While infected hosts not only transmit the virus to members of their same species, spillover can occur into other mammals in the surrounding area. For the most part, there remains a compartmentalization of specific variants within a geographic area, isolating virus populations and generating of very distinct genetic markers.[9,20] Monoclonal antibodies and FAT have identified that rabies isolates from unique geographic locations show a special reactivity patterns in the glycoprotein and nucleoprotein component of the virion.[24] Rabies isolates from Africa will therefore differ in their antigenic makeup from those of North America and Europe.[11,24] It is imperative that a rabies diagnostic tool be able to identify such differences in virus reactivity.

Further studies of rabies virus variants have determined the greatest difference in glycoprotein reactivity is found between bats and terrestrial species.[7,9] Therefore, there is a distinguishable difference between virus variants hosted by vampire bats in Latin America and skunks and raccoons in North America. It is suspected that nearly all of the cases from this study were obtained from a similar geographic population, and therefore most likely were infected with common North-American virus variants. It might be questioned then, whether IHC can successfully be applied in suspected tissue samples in Latin America, where a different variant is found. Impressively, previous use of this protocol in bovine cases in Brazil successfully detected rabies antigen, demonstrating this polyclonal antibody's applicability to the widest range of rabies virus variants.[19]

IHC procedures have also been successfully applied throughout North America, Africa and the Far East for rabies-antigen in FFPE sections.[11,12] An IHC technique used in South Africa identified both viverrid rabies-virus antigen and canid rabies-virus antigen in species such as the yellow mongoose (*Cynictus penicillata*), domestic dog, black-backed jackal (*Canis mesomelas*) and bat-eared fox (*Otocyon megalotus*).[11] Therefore, IHC is showing promise of becoming a universal test for detecting rabies.

Not only does this antibody identify such variants, but it is commercially available for purchase by any diagnostician. Being able to buy a commercially-made primary antibody greatly reduces the cost and labor required in many laboratories to make their own. Most experimenters using IHC still rely on using animal lines, such as rabbits or guinea pigs, to generate primary antibodies, or must have them donated by universities or research centers. [6,11,15,22] An immunohistochemical study of human rabies recently done in 2000 notes that specific anti-rabies antibodies were not yet commercially available,[8] and this new aspect of IHC provides numerous

advantages. Increasing the accessibility that diagnostic labs in developing countries have to such commercial IHC antibodies will greatly reduce costs required for their rabies surveillance.

IHC also overcomes many of the difficulties in acquiring fresh samples necessary for FAT, which contain live virus and any handling is highly dangerous.[27] In formalin-fixed specimens used in IHC, the rabies virus is rapidly inactivated by formaldehyde, making the transport and laboratory processing of these tissues much safer.[11] FAT analysis also requires the use of expensive ultraviolet light microscopes, which may be difficult for many diagnostic facilities to afford.[12] There is only a limited time of about 6 months for fresh brain to be used for FAT testing, and the impermanent fluorochrome dyes fade very quickly after the test is administered, limiting further histological examinations.[1,11] However, the hematoxylin staining used in IHC does not fade and can be used for permanent records and retrospective studies, and allows for the assessment of viral antigen and histopathological lesions at the same time.[1,11] Any rabies-negative case can be further examined for other causes of the nervous signs described and encephalitis.[11,20]

In developing countries in tropical regions, high temperatures hinder the collection and preservation of fresh specimens.[12] Fresh brains must be examined within a few hours with FAT to get the most reliable results and the success lies in the quick transport of samples to the lab under cold conditions.[1] In many situations, an entire brain cannot easily be transported to the diagnostic laboratory due to the inconvenience of storing and high expenses, especially for large livestock. Therefore, portions of the brain need to be selectively removed in the field.[2,7] More commonly in Latin America, Africa and China, field collections of brain samples occur distant from diagnostic laboratories, causing widespread underreporting of the disease.[12] Sampling must be based on a knowledge of preferred brain sections in order to ensure that the small sample that

makes its way back to the lab is reliable. Delays in sample collection and shipping in some countries may add 5 or more days from the death of the animal to available laboratory test results which can be very disadvantageous.[7] With any delay in rabies diagnosis in humans, the number of contacts requiring PEP will increase, which is on average around 50 people per case.[27] In countries with strong rabies control programs, especially in North America, the disease is uncommon in domestic animals, so an apparently healthy animal can simply be confined and observed daily for 10 days for clinical signs before PEP is administered to a human.[24] Unfortunately in other countries where rabies is poorly controlled in domestic animals, treatment mush begin right after exposure and rapid results are even more in demand.[7,20]

Even with attempts at improving rabies control programs outside of the United States, 99% of all human rabies deaths are still occurring in the developing world and high rates of transmission from domestic dogs, especially in Africa and Asia, has generated a global burden of rabies.[7,21,24] Rapid population growth and overcrowding in urban areas, poor infrastructure and sanitation, inadequate funds and equipment, unsuitable public education, and a shortage of experienced personnel have all hindered canine rabies control.[21,24] The annual incidence of rabies per 100,000 persons in India is 0.37 in urban settings and 2.49 rural towns. China's incidence rates are 0.29 and 0.15 for urban and rural communities, respectively, and Africa's rates lead with a 2.00 incidence in urban areas and 3.60 in rural areas per 100,000 persons.[7,22] Despite such data from reported cases in these countries, accurate estimates of human rabies deaths are impossible to obtain because local laboratories are inadequate or non-existent for the systematic detection of both human and animal rabies cases.[7] The World Health Organization estimated in 1999 that the proportion of rabies cases detected and reported represents only 3% of the total

global rabies mortality.[7] In Africa, diagnostic confirmation of rabies was available for less than 0.5% of estimated human cases, making rabies' true public health impact very underreported.[7,27]

Rabies surveillance can be greatly enhanced through an increased capacity to conduct reliable diagnostic testing on-site in localities, rather than through the current setup of central diagnostic laboratories due to the expense and expertise demanded by FAT and the fluorescent microscope.[7] The absence of an affordable confirmatory test in these countries, combined with the unavailability of human rabies vaccines creates the greatest demand for quick PEP administration.[12] Considering that more than 7 million people will be potentially exposed to rabies every year, research and advancement in diagnosis must continue to focus on human death prevention and obtaining reliable test results as fast as possible.[7] Rabies diagnostic tests done post-mortem of animals that have bitten a person make up the most important diagnostic contribution to the control and prevention of rabies worldwide.[7] The most detrimental diagnostic error would involve false-negative results of an animal that has bitten a human, which leaves out proper PEP administration and might lead to death. Consequences of a false-positive can prompt unnecessary PEP treatment and misleading epizootiologic data as well. As tests such as IHC and FAT continue to become more available, they still must maintain a level of 100% sensitivity in order to be considered as the primary tool in facilitating human exposure determinations.[7]

Immunohistochemistry provides an alternative solution in which laboratories worldwide can apply a commercial polyclonal antibody to specific parts of the brain most reliable for detection, and rapidly diagnose rabies in any mammal that has possibly exposed a human to this serious disease.

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