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Rabies Necropsy Techniques in Large and Small Animals

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TITLE:

Rabies Necropsy Techniques in Large and Small Animals

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KEYWORDS:

rabies, animal, necropsy, technique, safety, virology

SHORT ABSTRACT:

The goal of this protocol is to demonstrate safe necropsy techniques in small and large animals to obtain satisfactory tissue samples for rabies testing.

LONG ABSTRACT:

The New York State Department of Health (NYSDOH) Rabies Laboratory receives between 6,000 to 9,000 specimens annually and performs rabies testing for the entire state, with the exception of New York City. The Rabies laboratory necropsies a variety of animals ranging in size from bats to bovids. Most of these specimens are animals exhibiting neurological signs, however, less than 10% actually test positive for rabies; implying trauma, lesions or other infectious agents as the cause of these symptoms. Due to the risk of aerosolizing undiagnosed infectious agents, the Rabies Laboratory does not use power tools or saws. Three necropsy techniques will be presented for animals whose skulls are impenetrable with scissors. The laboratory has implemented these techniques to decrease potential exposure to infectious agents, eliminate unnecessary manipulation of the specimen and reduce processing time. The advantages of a preferred technique opposed to another is subject to the trained individual processing the specimen.

INTRODUCTION:

Working on the necropsy floor of a rabies laboratory is inherently dangerous. At times, specimens arrive with embedded porcupine quills, foreign objects including arrows/bullets/pellets or exposed bone shards that may penetrate the protective shipping wrap. Improper packaging can result in leakage, endangering individuals who unpack specimens. In addition to physical injury, necropsy technicians risk exposure to unknown zoonotic infectious agents from the CNS and

body fluids of the specimens. Additionally, ectoparasites carried by the specimen may transmit other zoonotic diseases, as fleas and ticks are commonly seen on submitted animals. Depending on geographic location and species involved the diseases exposed vary. Arboviruses such as Eastern equine encephalitis virus (EEEV) or West Nile virus (WNV), tick-borne diseases including Lyme disease or tularemia, bacteria causing Q fever or tuberculosis, and infectious prions name a small number of the possible dangers[1-3].

The purpose of these methods is to demonstrate safe and efficient necropsy techniques using instruments that minimize the potential for aerosolization unlike power tools or saws[4,5]. Commonly, the necropsy of small animals in the rabies laboratory requires cutting away the cranial muscles and using a hammer and chisel to open the caudal dorsal portion of the calvarium[6]. Removing this area of calvarium exposes the hind brain, including the entire cerebellum and cranial brain stem. Modified necropsy techniques may be performed on the ventral part of the skull, avoiding the large cranial muscles and thicker regions of the skull. However, these modified necropsy techniques are only possible when the specimen is without cervical vertebrae.

Similarly, brain tissue in large animals can be removed by separating the cranial muscles and opening the caudal dorsal portion of the skull[7]. Considerable effort is required to expose the cerebellum and brain stem as the skulls of larger animals are generally thicker. To avoid penetrating the skull, the head of a large animal is positioned so the ventro-caudal portion of the skull is facing the technician. Using modified instruments, the cerebellum and brain stem are removed through the foramen magnum. This is similar to the sample acquisition method recommended by the TSE European Union Reference Laboratory for Transmissible Spongiform Encephalopathy (TSE) investigations[8]. Cranial vertebrae should be removed beforehand to provide access to the foramen magnum.

Application of these techniques are beneficial to suitably trained technicians in rabies laboratories. As the rabies laboratory receives samples of various sizes, from juvenile bats to adult draft horses[9], the technician has several methods to choose from based on the individual circumstance. The method demonstrated for a large animal is also appropriate for veterinarians who perform necropsies in the field, since shipping an entire large animal head for rabies testing is cumbersome and costly. Implementing any of these techniques will improve safety by decreasing the potential of aerosol production, reduce the handling of the specimen and save processing time. However, as the field does not have the same advantages as a laboratory set up specific for rabies testing, it is essential that any modifications made to these procedures focus on safety, especially the use of personal protective equipment (PPE).

PROTOCOL:

All methods described were approved by the Wadsworth Center Institutional Animal Care and Use Committee (IACUC).

1. Preparation

1.1. Don PPE, at minimum eye protection (glasses or face shield), surgical or N-95 mask, and non-latex gloves.

1.2. Prepare work area, ideally a bio-safety cabinet (BSC), with a disposable work surface covering (e.g., kraft paper or absorbent pads) and clean necropsy instruments (**Figure 1**).

1.3. Place the specimen on the work surface and use instruments to manipulate it to assess condition of the sample including evidence of decomposition, damage to skull, potential hazards (e.g., porcupine quills, scalpel blades), and the quality of the decapitation.

2. Ventral method

NOTE: When the specimen is properly decapitated at the jaw-line, the foramen magnum and the occipital condyle will be exposed. The ventral method is less complicated for retrieving the cerebellum and brain stem.

2.1. Position the specimen with ventral side up and nose directing distally toward the back of BSC.

2.2. Hold an orthopedic hammer/mallet in right hand (if right-handed) and at same time hold a councilmen chisel in left hand.

2.3. Position the chisel at a 45° angle with the corner point of the chisel directing between the right side of temporal bone and occipital bone making a “V” opening.

2.4. Strike the top of the chisel with the hammer until the two bones separate. Make the cut to adjacent to the basisphenoid bone.

2.5. Repeat on the left side of temporal bone/occipital bone (**Figure 2A**).

2.6. Bend the “V” area of skull downward with the chisel. Expose the entire rhombencephalon area of the brain (cerebellum and brain stem) (**Figure 2B**).

2.7. Scoop out the brain stem and cerebellum with scissors and forceps. Remove any remaining pieces from the skull if the brain stem and cerebellum did not come out in a single piece.

3. Dorsal method

NOTE: If the specimen has a poor decapitation (foramen magnum not visible) and the neck cannot be easily removed during necropsy or if damage to the cerebellum is suspected, the dorsal method should be utilized.

3.1. Position the specimen dorsally with the nose directing distally toward the back of BSC.

3.2. Using tumor tenacula, grasp the left temporal muscle with teeth of tenacula and lock by squeezing the handle.

3.3. Cut the temporal muscle down to bone with sharp carving knife.

3.4. Rotate the specimen 180° with tenacula and knife (not hand) and repeat the process on the opposite temporal muscle. Expose the skull.

3.5. Position a chisel at a 45° angle with the corner point of the chisel on the center of the skull at the juncture of the parietal and intraparietal bone.

3.6. Strike the top of the chisel with a hammer until a horizontal opening is made on the top half of the skull at parietal bone.

3.7. Rotate the specimen 180° and repeat the process on the opposite side.

3.8. Insert the point of the chisel into the end of cut 1 (**Figure 3A**) and at 90° of horizontal opening. Strike with the hammer until the opening reaches occipital bone (approximately 10 cm depending upon size of the specimen).

3.9. Roll the specimen and repeat on the opposite side at the end of cut 2.

NOTE: With specimen dorsal and nose positioned toward the back of BSC, the openings in the skull resemble an upside-down "U".

3.10. Insert the teeth of the tenacula into the skull at the bottom of the "U" and pry towards oneself. Expose the caudal end of the cerebrum and the cerebellum (**Figure 3B**).

3.11. Use scissors as a scoop and pry out entire cerebellum from within the cavity.

3.12. Use tissue forceps to tease out the brain stem from the foramen.

4. Large animal method

4.1. Position the specimen so that the dorsal part of the skull is in contact with the necropsy surface with the caudal portion of the skull and foramen magnum facing the technician.

4.2. Insert the modified stiletto knife into the foramen magnum in between the spinal cord and spinal meninges as far as possible.

4.3. Score around the spinal cord to separate the cerebellum and brain stem from the spinal meninges. After the knife is inserted through the foramen magnum, gently angle the knife to follow along the skull as much as possible.

4.4. Insert a chemistry spatula or thin, long handled spoon into the space between the neural tissue and spinal meninges.

4.5. Probe around the spinal cord and cerebellum to ensure the connection to the spinal meninges have been severed.

4.6. Hold the brain stem with forceps. With the other hand, advance the spoon rostrally then dorsally to scoop up the cerebellum. Simultaneously pull back on the brain stem with the forceps and scoop out the cerebellum using the spoon.

NOTE: It may take more than one attempt to recover adequate cerebellum for rabies testing.

5. Post necropsy

5.1. Dispose of all disposable materials (gloves, pads, work area coverings) and unused tissues in biohazardous waste.

5.2. Clean and disinfect all instruments with method available (e.g., industrial dishwasher, autoclave, chemical disinfectant, boiling).

5.3. Clean and disinfect all work surfaces with 20% bleach and/or 70% ethanol.

REPRESENTATIVE RESULTS:

All terrestrial samples submitted with skulls between January 31, 2019 and February 28, 2019 had information regarding the presence of a neck and the method of necropsy collected. During that time, 170 heads were necropsied with 18 species represented. 52% (89/170) were properly decapitated. The remaining had at least one vertebra attached including three whole body specimens. The ventral method was used 75% (128/170) of the time, of those, necks were present on 49. Specimens submitted with a neck will have it removed during necropsy to allow for the ventral method whenever possible. Three large animals (cow, deer, pig) were submitted and in two cases the large animal protocol was used. The large animal protocol was not used on the pig because extra brain tissue samples were required for additional testing. A squirrel was submitted with a crushed skull and simply cutting away the skin exposed brain tissue, thus none of the above methods were used (**Table 1**).

On fresh intact submissions, all three methods will result with the required tissue for reliable rabies diagnostic test results. Occasionally, the cerebellum and brain stem cannot be removed intact, although after removing all tissue from the hindbrain these tissues can be identified and processed accordingly.

These three valuable methods cannot compensate for poor specimen quality caused prior to receipt at the lab. Trauma, decomposition and poor decapitation methods can affect the outcome regardless of how efficiently the samples are collected.

FIGURE AND TABLE LEGENDS:

Figure 1: Instruments used in rabies necropsy. Curved sharp-blunt mayo scissors, smooth-tipped tissue dressing forceps without teeth, councilman orthopedic bone chisel, orthopedic mallet-hammer, locking tumor-tenacula, restaurant-quality carving knife, modified stiletto knife, chemistry spoon, and sharpened tablespoon.

Figure 2: Ventral method of necropsy. (**A**) Location of cuts: Place point of a chisel at base of arrow, cut in direction of green arrow and repeat following the yellow arrow forming a "V" around the foramen magnum. Pry "V" down to expose the brain stem and cerebellum. (**B**) Brain stem (green) and cerebellum (blue) when exposed using the ventral method of necropsy.

Figure 3: Dorsal method of necropsy. (**A**) Location of cuts: Place point of a chisel at base of arrow and cut in direction of arrow in the order noted forming a "U". Pry "U" down to expose the cerebellum with the brain stem below it. (**B**) Cerebellum (circled) when exposed using the dorsal method. Brain stem lies directly beneath and is not visible until cerebellum is removed.

Table 1: Breakdown of specimens requiring tissue removal from the skull submitted from January 31, 2019 through February 28, 2019 at the New York State Department of Health Rabies Laboratory.

DISCUSSION:

Specimens submitted for rabies necropsy often have a history of clinical signs compatible with a neurological illness. The presence of clinical illness may be associated with a variety of disorders, including zoonotic diseases, increasing the risk to staff of a laboratory acquired infection. To reduce these risks, techniques have been implemented that decrease the handling and manipulation of specimens.

The methods demonstrated represent a necropsy event to remove desired tissues from a single animal only. More commonly multiple specimens are processed in a shift and care is needed to ensure no cross contamination between samples. A clean worksurface (disposable kraft paper or pads), a new set of clean, disinfected instruments, and glove changes are mandatory. Once tissues are obtained, the individual laboratory's protocol for processing can be followed, including making slides for microscopy or RNA extraction for molecular methods.

There are several essential prerequisites for successfully implementing these techniques in the laboratory or field. Prior rabies vaccination and PPE are critical for anyone necropsying a rabies suspect animal. Individuals working in rabies laboratories should have their serum tested every six months to ensure an adequate level of anti-rabies antibodies are present[10]. It is important to remember that other zoonotic diseases, such as EEEV, WNV and Bovine Spongiform Encephalopathy (BSE), present similar signs as rabies and may also occur in rabies suspect animals[11,12].

Appropriate well-maintained instruments are essential to safely perform necropsy. Once a

specimen has been removed from its biohazard bag, it should only be manipulated with instruments, not hands, to decrease the potential for accidents. Prior to small animal necropsy, the technician should evaluate the condition of the specimen to determine whether a preferred ventral approach through the base of the skull is possible. With large animals it may be too cumbersome to fully evaluate the condition of the specimen as additional vertebrae may need to be removed before retrieving tissue through the foramen magnum.

Limitations present themselves in all necropsy techniques including specimen condition, tissue quality and the amount of remaining cervical vertebrae. The cervical vertebrae will not affect the results of rabies analysis, but severely decomposed tissue may result in unsatisfactory results. More sensitive molecular methods in rabies diagnostics may allow successful testing in certain samples unable to be tested by direct fluorescent antibody assay (DFA), including severely decomposed specimens[13]. However, no amount of sensitivity can replace the need for proper tissue sampling.

A common problem in the rabies laboratory is receiving inappropriate or inadequate brain tissue for testing when large animal necropsies are performed in the field. Without the required tissue, and if additional tissues are unavailable for resubmission, our rabies laboratory will perform testing on the tissue available but is unable to verify the specimen negative, instead it is unsatisfactory for testing. There are other published methods for field tissue collections such as the straw method or retro orbital route[14]. Both methods collect brain tissue without the need to open the skull. A straw or disposable pipette is inserted either through the foramen magnum or a hole created in the eye socket and pushed through the brain, essentially taking a core sample and not necessarily sampling the full cross section of the brain stem. As these field methods do not collect samples in a manner to be considered satisfactory for testing in our laboratory, these processes are not demonstrated or explored in this paper.

In the field large animal necropsy can be challenging for individuals who are not trained to remove the correct tissue for rabies testing. Instead the entire head of the animal, which can weigh between 20-45 kg, is submitted creating cumbersome transport for both the field veterinarian and rabies laboratory technicians. Frequent requests for training on large animal necropsy technique have been made to our laboratory. The objective of this manuscript is to distribute this information to individuals and groups whose work can benefit from these techniques.

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DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

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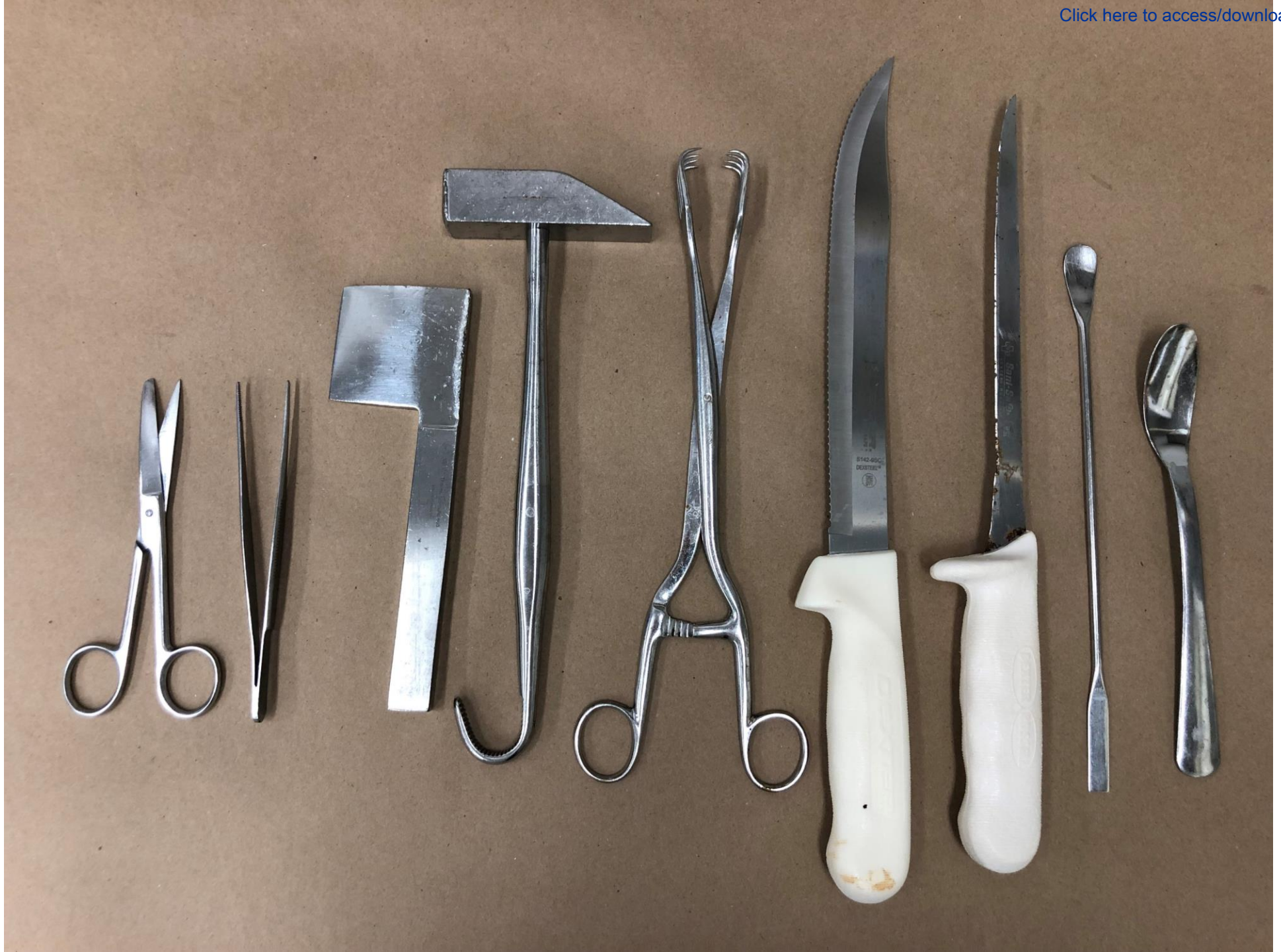

Figure 2A

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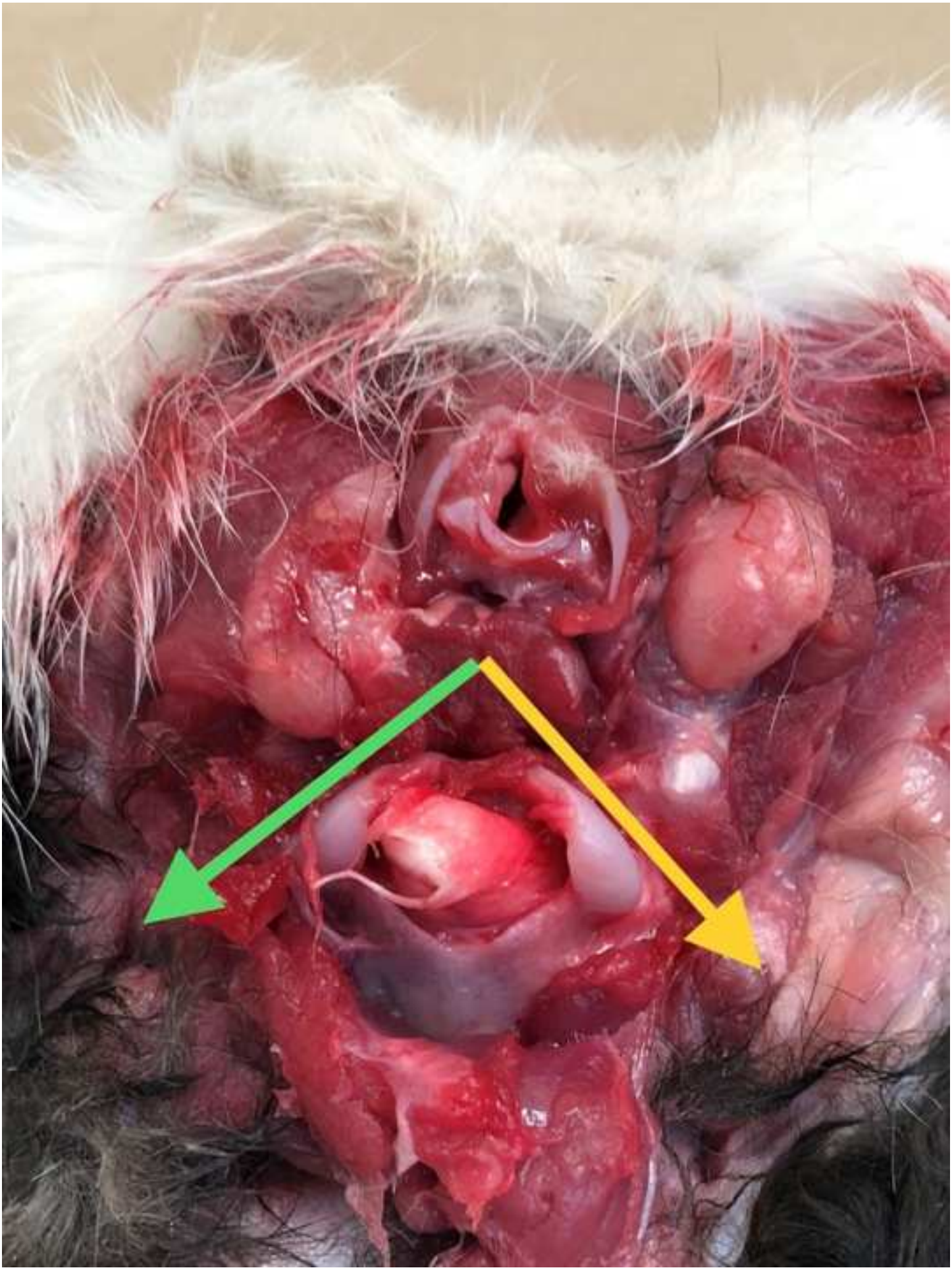

Figure 2B

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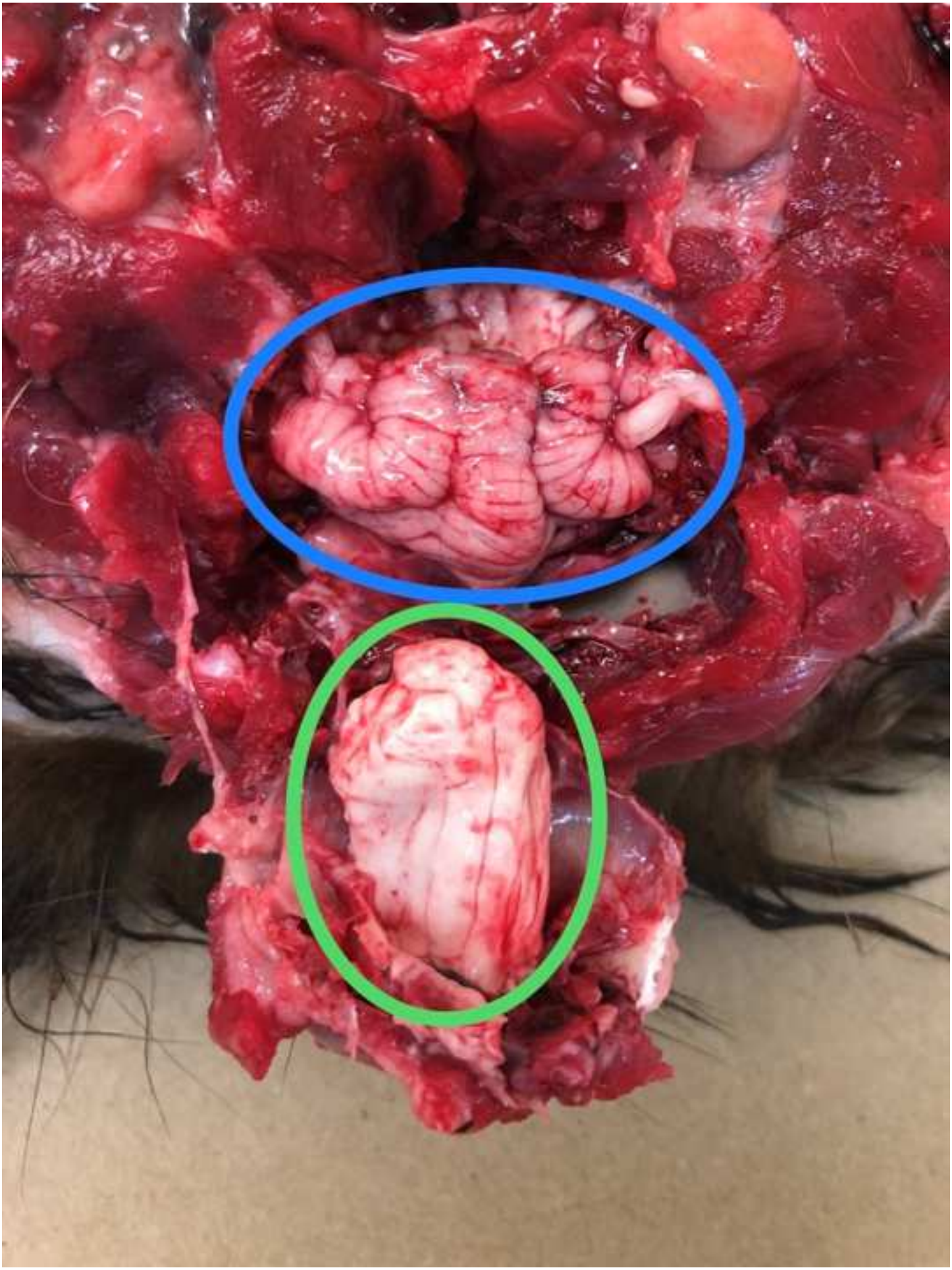

Figure 3A

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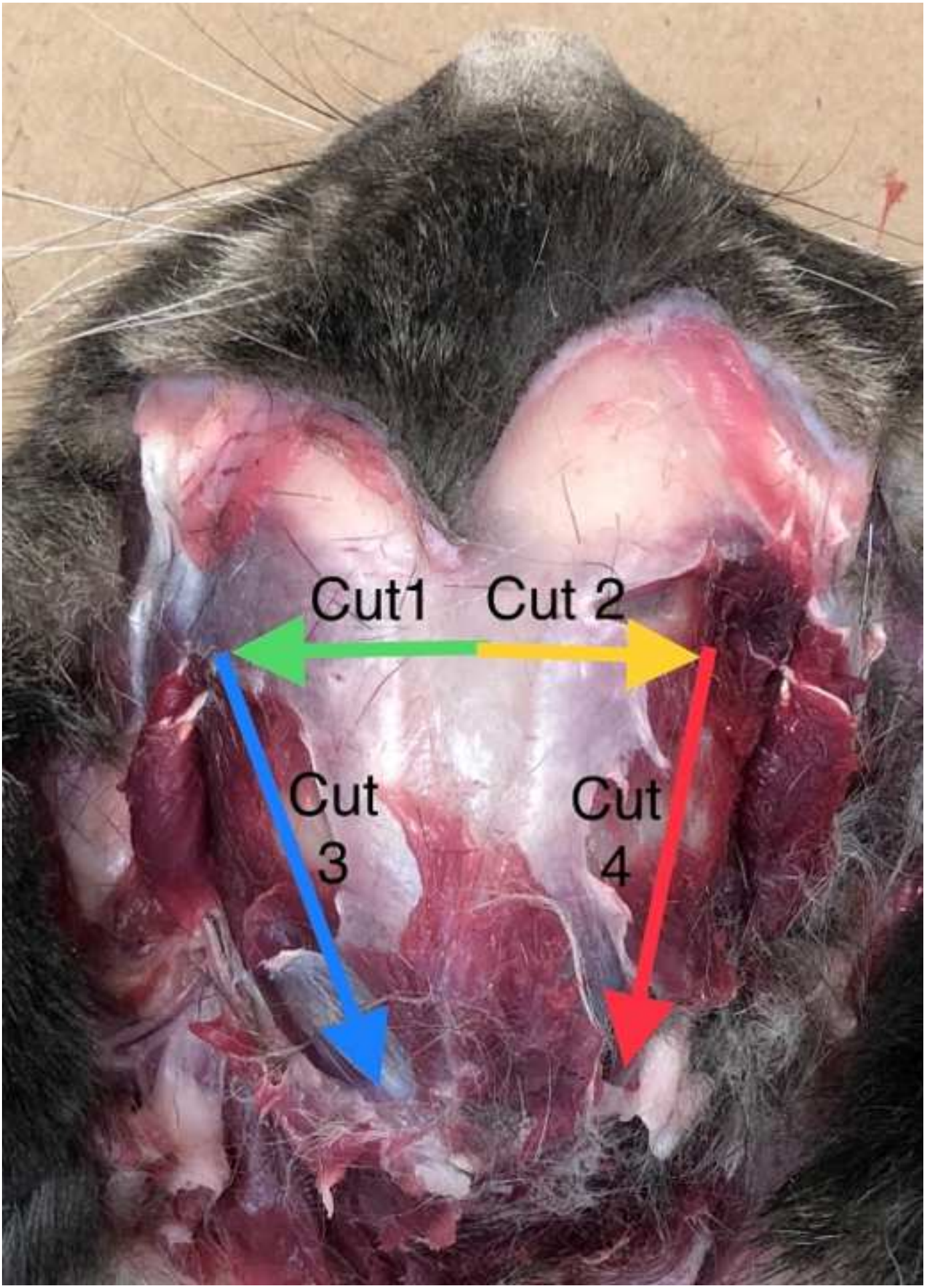

Figure 3B

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Table

species	vertebrae attached	method used V=ventral, D=dorsal, LA=large animal	total	comments
bear	no	V	2	
cat	no	V	32	2 small enough to open skull with scissors, 1 had abcess that was being investigated
cat	no	D	3	exposing top of skull
cat	yes	V	11	
cat	yes	D	8	
cow	no	LA	1	
coyote	no	V	1	
coyote	yes	V	2	
coyote	yes	D	3	
deer	no	LA	1	
dog	no	V	19	
dog	no	D	3	
dog	yes	V	2	1 was small dog
dog	yes	D	18	
ferret	no	V	1	
fisher	no	V	1	
flying squirrel	yes	D	1	whole body
grey fox	no	V	2	
grey fox	yes	V	4	
pig	yes	V	1	
porcupine	no	V	1	
raccoon	no	V	16	
raccoon	no	D	1	frozen
raccoon	yes	V	26	
red fox	no	V	2	
skunk	no	V	1	
skunk	yes	V	3	
squirrel	no	V	1	

squirrel	yes	D	1	full body, crushed skull, used scissors to cut away skin to exposed brain cavity
weasle	no	D	1	
woodchuck	yes	D	1	whole body
Total			170	

Breakdown of total

with neck	81
no neck	89
ventral method	128
dorsal method	39
large animal method	2
other method	1
ventral method with neck	49

Name of Material/Equipment	Company
Chemistry spoon	Any
Curved, sharp-blunt mayo scissors	Sklar
Large sharp restaurant-quality carving knife	Dexter
Locking tumor-tenacula	Diamond Scientific and Surgicals
Modified stiletto knife (6.5 inch long blade carving knife ground to 0.5 inch wide)	Dexter
Orthopedic mallet-hammer	Mortech
Sharp councilman orthopedic bone chisel	Shandon
Sharpened tablespoon or other long handled spoon	Any
Smooth-tipped tissue dressing forceps without teeth	Shandon
Powder-free non-latex gloves	Any
Safety glasses, goggles, or faceshield	Any
Surgery or N-95 mask	Any
Kraft paper, butcher paper, absorbent pad, etc	Any

Catalog Number	Comments/Description
14-2055	Sklar Operating Scissors 5-1/2 Inch Premium OR Grade Stainless Steel Finger Ring Handle Curved Sharp/Blunt
P94848	8" Scalloped Utility Knife, white handle
N/A	Czerny Tenaculum Forcep
P94848	Modified 8" Scalloped Utility Knife, white handle
N/A	Postmortem hammer with hook
60-5	Councilman's Chisel Blade: 2 in x 2.25 in standard 7 in
63-03	Shandon Broad Point Dressing Thumb Forceps

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Editorial comments:

Changes to be made by the author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Done

2. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

Added “All methods described were approved by the Wadsworth Center Institutional Animal Care and Use Committee (IACUC).” per Editor’s request, however this is not truthful. The animal specimens received for rabies testing are not alive therefore not covered by IACUC. We do have an IACUC approved protocol for the cases we do receive a live bat, but bat necropsy is not covered in this manuscript.

3. Please revise the Protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Done

4. Protocol: Please revise to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “NOTE.” Please include all safety procedures and use of hoods, etc.

Completed changing to Imperative tense and avoiding listed phrases. Added to protocol Preparation section which includes safety.

5. Line 302: Should be Figure 4.

Done

6. Table of Materials: Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol.

Done

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
Excellent manuscript describing a technique of broad interest.
The authors have extensive experience with the technique and are frequently asked to share these data.
A video format would be an excellent way of transmitting the technique

Thank you. Yes, we are frequently asked about sample collection from Veterinarians around the state, specifically for large animals.

Major Concerns:
Should include a brief description of how the gathered tissue will subsequently be prepared for analysis.

As different labs prepare their samples differently (impression vs. slip smears, extraction methods) preparation for analysis was not expanded on. Added to the "Once tissues are obtained, the individual laboratory's protocol for processing can be followed, including making slides for fluorescent microscopy or RNA extraction for molecular methods."

Might also include a line or 2 on how molecular assessment might impact the extraction technique.

NYSDOH Rabies Laboratory follows strict guidelines on acceptable tissue which does not differ between DFA and molecular techniques. A full cross section of brain stem and samples from all 3 lobes of the cerebellum or hippocampus are required and brain extraction techniques do not differ.

Minor Concerns:
Figure legend "5"is actually for figure "4"

Done

Reviewer #2:

Manuscript Summary:
Authors describe three different methods to collect samples from the central nervous system of large and small animals submitted for rabies diagnosis. The manuscript is suitable for publication in JoVE, although a few concerns need to be addressed. Great emphasis has been put on the potential hazards and risks laboratory staff are exposed to, overlooking however to discuss about the possible risk for cross-contamination of diagnostic samples in case of inaccurate necropsy procedures. Information on possible decontamination steps of utensils and necropsy table should have been addressed, by taking into consideration the new methods that are now encountered as gold standards (i.e. RT-PCR) and the relative risk for nucleic acid cross-contamination. Considering the worldwide distribution of the disease, moving the focus from US to a wider epidemiological context is strongly suggested, as well.

Our initial target audience was the veterinarian collecting samples themselves for submissions instead of submitting the full head. In those cases, the veterinarian is only collecting samples from one animal and the risk of cross contamination is not a concern. Other rabies laboratories in the US have asked about our techniques as well, hence our including the ventral and dorsal methods and my assumption that they already have techniques in place to avoid cross-contamination. I do agree that decontamination steps should be addressed and a section for post necropsy was added to the protocol.

We chose to keep the focus more on the US realizing that other countries with limited resources may use techniques that differ from ours and that our laboratory would not feel comfortable doing or reporting results using tissue samples that do not fit our requirements based on **Protocol for Postmortem Diagnosis of Rabies in Animals by Direct Fluorescent Antibody Testing** A Minimum Standard for Rabies Diagnosis in the United States https://www.google.com/url?sa=t&rct=j&q=&esrc=s&source=web&cd=1&cad=rja&uact=8&ved=2ahUKEwivi-L-yenhAhWJd98KHY6YCcwQFjAAegQIARAC&url=https%3A%2F%2Fwww.cdc.gov%2Frabies%2Fpdf%2Frabiesdfaspv2.pdf&usg=AOvVaw0FdUqrD2-kSVqvPQXh-IjA

Major Concerns:

Introduction:
Lines 48-54. Submitted animals could have been infected by (or even be drivers of … through different parasites) other infectious diseases with zoonotic potential: Tick-Borne Encephalitis virus, Rift Valley Fever, rickettsiosis are few examples. Thus, opening a carcass always poses a high risk for humans, a matter which should have been acknowledged by the authors.

We had touched on other zoonotic diseases in the introduction, but only mentioning arboviruses. More has been added about zoonotic disease potential.

Lines 70-72. The Authors should provide a reference regarding the removal of brainstem for TSEs investigation. This procedure was developed to allow vets and/or technicians to remove cerebellum and brainstem through foramen magnum at slaughterhouse or in the field by means of a modified spoon: https://protect2.fireeye.com/url?k=09f00f8d-55d40ec4-09f2f6b8-0cc47aa8d394-3f805f032cc98df8&u=https://science.vla.gov.uk/tse-lab-net/documents/tse-oie-rl-samp.pdf

Thank you for this link. I had not come upon it in my previous journal searches. I have included a reference to it.

Line 131. Include visible ectoparasites such as ticks.

Ticks and fleas were added to the introduction.

Discussion:
Lines 339-347. Since rabies infection is to be investigated, a careful evaluation of the test

feasibility is required before declaring the specimen unsatisfactory for diagnostic evaluation. In certain cases, animals might be linked to a human exposure. The recent revision of the OIE Manual for diagnostic tests includes molecular methods jointly with the recognized antigen based methods (namely the fluorescent antibody test and the direct immunochemical test) as gold standard for post-mortem diagnosis of animal rabies. There is some evidence which shows that molecular methods are more appropriate than antigen-based methods to detect viral RNA in putrefied samples (see Markotter, et al.,2015. doi: 10.4102/jsava.v86i1.1220. ; McElhinney et al., 2014. doi: 10.1016/j.jviromet.2014.06.024. ; Aiello et al, 2016. doi: 10.1016/j.jviromet.2016.03.017. Prabhu et al., 2018. doi: 10.3390/vetsci5010024.). I would therefore suggest rephrasing this part by addressing the above observations.

Some more has been added to the discussion regarding this topic. Our laboratory has recently published the paper https://doi.org/10.1177%2F0033354918810776 **Clarifying Indeterminate Results on the Rabies Direct Fluorescent Antibody Test Using Real-Time Reverse Transcriptase Polymerase Chain Reaction**

Minor Concerns:
Few minor typing errors appear along the text. Consider accurate revision. Table 1 appears not in line with the scope of the journal.

Previously we received the following comment from the editor and is the reason for Table 1 "Please include a figure or a table in the Representative Results showing the effectiveness of your technique **backed up with data**."

Reviewer #3:

Manuscript Summary:
In their manuscript entitled "Rabies Necropsy Techniques in Large and Small Animals", the authors described three different protocols for the collection of brain samples for the post-mortem diagnosis of animal rabies. The manuscript is well written and understandable, although this is a bit difficult without the video. I have only few minor comments below.

Thank you and agree that it is difficult to envision without the video.

Major Concerns:
No

Minor Concerns:

It could be of interest if the authors discuss about the other rapid techniques for brain samples collection applicable in the field and which are mentioned in WHO and OIE manuals on rabies (such as those using the retro-orbital and the occipital foramen route), especially because the authors indicate that some of their techniques can by applicable in such field conditions.

The other methods have been added to the discussion. They would be beneficial in field conditions in a surveillance capacity. However since the lab follows **Protocol for Postmortem**

Diagnosis of Rabies in Animals by Direct Fluorescent Antibody Testing A Minimum Standard for Rabies Diagnosis in the United States https://www.google.com/url?sa=t&rct=j&q=&esrc=s&source=web&cd=1&cad=rja&uact=8&ved=2ahUKEwivi-L-yenhAhWJd98KHY6YCcwQFjAAegQIARAC&url=https%3A%2F%2Fwww.cdc.gov%2Frabies%2Fpdf%2Frabiesdfaspv2.pdf&usg=AOvVaw0FdUqrD2-kSVqvPQXh-IjA any laboratory in the United States would not be able to use samples collected in that manner, especially in the case of a human exposure. More information about our required samples is included in the response to your final concern.

- Lines 41-43 and general comment about the dorsal method: Maybe I did not understand well the technical description of dorsal method without the video but it seems that this is the classical method used by most of the rabies laboratory worldwide, and for which the description is available from previous OIE or WHO manuals on rabies. So I did not identify the "development" made by the authors for this technique.

Agree that “developed” is the incorrect word and replaced with “implemented”.

- Line 273: No capital for "Squirrel"

Done

- Lines 325-227: Add corresponding reference(s).

Done

- Lines 327-329: Precise this statement and the related diseases (especially EEEV) depend on the geographical contexts and that other pathogens can have the same clinical pictures in different settings than the US.

Added in the introduction that other diseases that may affect the animal vary by geographic location and included more examples.

- Line 302: Replace "Figure 5" with "Figure 4".

Done

- Lines 343-347: I assume that this statement is the one use only at the NYSDOH rabies laboratory with the technique described in the article. If so, it needs to be mentioned. Indeed, the best brain tissue samples for the post-mortem diagnosis of animal rabies based on FAT are brain-stem and/or cerebellum, which are recommended by OIE and WHO to ensure high sensitivity of the test. However, it is not specified that these samples are satisfactory only if a full cross section of the brain stem is done and a portion of all three lobes of the cerebellum, which is very stringent and limiting.

NYSDOH Rabies Laboratory follows **Protocol for Postmortem Diagnosis of Rabies in Animals by Direct Fluorescent Antibody Testing** A Minimum Standard for Rabies Diagnosis in the United

States https://www.google.com/url?sa=t&rct=j&q=&esrc=s&source=web&cd=1&cad=rja&uact=8&ved=2ahUKEwivi-L-yenhAhWJd98KHY6YCcwQFjAAegQIARAC&url=https%3A%2F%2Fwww.cdc.gov%2Frabies%2Fpdf%2Frabiesdfaspv2.pdf&usg=AOvVaw0FdUqrD2-kSVqvPQXh-IjA

In which it states that a full cross section of brain stem is required due to unilateral flow of rabies virus in the CNS, and we have also seen that in our laboratory in an unpublished study. It recommends that a full cross section of cerebellum be used, however in the case that all the lobes are not available it can still be acceptable. The more stringent lab specific requirement of a sample of all three lobes has been removed and replaced with the compendium's requirement.