Collection of brain stem for post-mortem diagnosis of rabies in animals by the LN34 pan-lyssavirus real-time RT-PCR assay

Crystal M. Gigante,Rene Edgar Condori,Lillian Orciari,Todd G. Smith,Yu Li

Abstract

Patterns of rabies virus spread within the central nervous system suggest that a thorough examination of the brain stem is critical for rabies diagnosis. Viral RNA is widespread in the brain of most animals positive for rabies. However, because virus spread may be unilateral, especially in larger animals, a negative finding for rabies can be made only if a complete cross section of the brain stem is examined. Examination may be made at the level of the pons, medulla, or midbrain of the brain stem.

While a negative finding for rabies can be made only if a full cross section of brain stem tissue is examined, incomplete or suboptimal specimens should be tested, if possible. Identification of rabies virus RNA in any tissue is diagnostic of rabies infection [1, 2]. Examples of samples that may be tested for rabies virus RNA to rule-in (but not rule-out) rabies infection are hippocampus, cerebellum, cortex, saliva, and nuchal skin biopsy.

Citation: Crystal M. Gigante,Rene Edgar Condori,Lillian Orciari,Todd G. Smith,Yu Li Collection of brain stem for post-mortem diagnosis of rabies in animals by the LN34 pan-lyssavirus real-time RT-PCR assay. **protocols.io**
dx.doi.org/10.17504/protocols.io.n4sdgwe
Published: 01 Jun 2018

Guidelines

Purpose

To describe the procedure of collecting brain stem tissue from a postmortem animal. The collected tissue can be used in the diagnosis of lyssavirus infection by the LN34 real-time RT-PCR assay.

Responsibility

Laboratorian will perform the procedure according to the protocol. Once sample collection is complete, the work area is disinfected, any biohazardous waste autoclaved or incinerated before discarding, and samples returned to storage.

Specimens

Postmortem animal brain tissue

Equipment, Materials and Reagents

1. Necropsy instruments
    - 1 set of instruments is needed per sample to prevent cross contamination between samples.
2. Autoclave and/or instrument sterilizer
    - All instruments should be cleaned and sterilized before reuse
3. Specimen storage containers
    - Container must be large enough for reserved portions of brain stem.
    - Because of the risk of breakage, glass vials and tubes are unacceptable for specimen storage.
4. Freezer
    - Long term storage should be at -70°C or lower
    - Frost-free freezers should not be used, as heat cycles in such freezers can compromise tissue integrity and, therefore, test results.
5. PPE:
    - heavy rubber gloves or other cut-resistant gloves
    - laboratory gown
    - waterproof apron
    - surgical mask
    - boots
    - protective sleeves
    - face shield
    - disposable gloves
6. Polyester fiber-tipped applicator swab
7. Scalpel
8. Sterile petri dish or paper towel
9. Disposable sterile screwtop microcentrifuge tubes with o-ring (2 ml)
    - In a clean room, add 1 ml TRIzol reagent to one tube for each sample.
    - Label tubes with corresponding accessioning information.
10. Acceptable surface decontaminants
    1. Quaternary ammonium disinfectant (1:256)
    2. 70% Isopropanol
    3. Iodinophors

Before start

Definitions and Keywords

BSC – Biological Safety Cabinet

PPE – Personal Protective Equipment

Room Temperature – Temperature range of 20°C to 25°C (68°F to 77°F)

Quality Control and Corrective Action

- All issues, comments, concerns, or deviations from the protocol are to be documented and brought to the attention of the lab supervisor or technical supervisor immediately if they are thought to affect the quality of the end product in any aspect.
- Temperature levels are to be monitored on applicable equipment to ensure incubators, refrigerators, and freezers stay within criteria set for acceptable temperature range.
- Specimen will be assigned based on the test ordered and/or the recommendations of the technical supervisor or lab supervisor.
- All samples are to have unique identifiers and corresponding accession labels provided in advance of starting the necropsy procedure.

Protocol

Shipment of samples

Step 1.

- Because rabies prophylaxis is usually delayed pending a laboratory report, specimen transit time to the laboratory should be as short as possible, preferably within 48 hours.
- A fresh, unfixed brain sample is necessary for rabies rule-out. Formalin-fixed brain tissue can be used for positive diagnosis using an RNA extraction protocol for formalin-fixed tissue (for example, RNeasy FFPE kit (Qiagen)).
- Refrigeration will preserve a sample for at least 48 hours.
- Repeated freeze-thaw cycles may reduce test sensitivity and should be avoided.
- Biocontainment during specimen transport is critical, to prevent both contamination of the outside of the package and cross-contamination between samples within the package

Sample sufficiency

Step 2.

- A qualitative assessment of the condition of each sample should be made upon arrival in the laboratory.
- A negative rabies diagnosis can only be made for sample that is representative of a full cross section of brain stem. If the condition of the tissue prevents the confident identification of brain stem tissue, the sample should be identified as unsatisfactory.
- In the case of unsatisfactory specimen, testing should still be performed. Positive test results are reported as such. If negative results are obtained on unsatisfactory tissue, the test report should state only that the condition of the sample is such that tests cannot rule-out the presence of rabies virus in the specimen. The negative results should not be mentioned, since

this is often misinterpreted as a negative diagnosis.

Sample processing

Step 3.

- Samples submitted to the laboratory may be a complete carcass (only for bats), an intact head, whole brain or dissected brain tissues.
- Dissected brain tissue must include a complete cross section of the brain stem.
- All material submitted with a sample, including the carcass, should be held frozen until the test is completed and results are reported.
- A single additional freeze-thaw cycle will have no effect on results if repeat testing of the sample should be required.
- Retention of the carcass is necessary to verify the identity of an animal in the case of unusual test results and to identify a wild animal to species.
- Unique sample identifiers (accession numbers) should be used to label boxes and all items accompanying the sample.

Sample handling

Step 4.

- All necropsy and tissue processing must include proper identification of each sample and avoidance of any practice that could lead to cross contamination of samples.
- Each specimen should be handled on a clean work surface with new disposable gloves.
- All instruments used during necropsy and dissection must be thoroughly disinfected by boiling or autoclaving followed by thorough washing before reuse.
- Instruments not in use should be kept in closed storage. Only those instruments in use for processing a single sample should be exposed.
- Surface decontamination of tissue samples for disposal with approved disinfectants is insufficient. Tissues samples must be autoclaved or incinerated to inactivate virus.

Sample retention

Step 5.

- Frozen reference material taken at necropsy should be retained in the laboratory for all test samples.
- It is recommended to retain test samples for a minimum of 2 to 6 months.
- Representative positive samples should be maintained for longer periods for use as controls, for epidemiologic typing, and for other purposes.
- Storage containers for reference material must be large enough that reserved portions of brain stem remain recognizable and allow complete cross sections to be made if repeat testing is required.

Collection of a cross section of brain stem

Step 6.

Set up your work space

- Frozen samples should be thawed just prior to testing.
- Clean and disinfect the work surface prior to starting work.
- Lay out plastic-lined absorbent pad(s) as a clean work surface.
- Place reagents, and supplies for the first sample only in the BSC or work area. Additional clean

materials and supplies should be stored outside the BSC or away from the work area and easily accessible.

Collection of a cross section of brain stem

Step 7.

Collect brain tissue representing a full cross section of brain stem, including both left and right sides.

1. The brain stem is anterior to the cerebellum and is continuous with the spinal cord. The uppermost portion of the brain stem is the midbrain; the hindbrain portion of the brain stem is composed of pons and medulla oblongata.
2. Tissue including a complete cross (transverse) section of at least one area of brain stem is necessary for rabies testing.
3. For small animals, the entire brain stem may be collected.
4. For larger animals, collection of a cross section is suggested.

NOTE: Cattle are often tested for both rabies and bovine spongiform encephalopathy (BSE). Tests for BSE require only the obex. Care should be taken so that sufficient brain stem tissue is reserved for rabies testing.

Collection of a cross section of brain stem

Step 8.

OPTIONAL: If performing DFA test, collect brain impressions at this point. The tissue remaining after collection of brain impressions for DFA can be used for RNA extraction and testing by the LN34 assay.

Prepare sample for RNA extraction

Step 9.

IMPORTANT: Once TRIzol reagent is added, samples can no longer be used for antigen-based detection methods. Whenever possible, retain a portion of original tissue without TRIzol reagent for future testing.

Prepare sample for RNA extraction

Step 10.

Add 200 mg of tissue (about the size of a pea or <250 µl liquid volume equivalent using volume approximations on tube) into a 2 mL screw-top microcentrifuge tube containing TRIzol reagent using the stick side polyester fiber tip applicator or similar sterile object.

Prepare sample for RNA extraction

Step 11.

1. For small to medium animals, place the entire piece of brain stem (>10 mg) into the tube containing 1 ml TRIzol reagent
2. Thoroughly homogenize brain pieces using a bead beater, micro tissue grinder or sterile applicator stick

NOTE: Homogenization and other suspension methods are likely to create aerosols and require the use of a BSC (class II).

Prepare sample for RNA extraction

Step 12.

For larger animals, the cross section of brain stem will be too large to test in its entirety.

Homogenize the whole cross section in TRIzol, then dilute:

1. Cut the cross section of brain stem into small pieces
2. Transfer all pieces to a tube containing TRIzol reagent
3. Thoroughly homogenize brain pieces using a bead beater, micro tissue grinder or sterile applicator stick
4. Add 200 µl homogenized tissue to 1 ml fresh TRIzol reagent
5. If the tissue is not homogenized well enough, the sample taken will not represent the entire brain stem and cannot be used to rule-out rabies.

NOTE: Homogenization and other suspension methods are likely to create aerosols and require the use of a BSC (class II).

Prepare sample for RNA extraction

Step 13.

Clean and disinfect the workstation, equipment, and outside of sample tubes.

Prepare sample for RNA extraction

Step 14.

Sample storage

- Samples stored in TRIzol can be immediately processed for RNA extraction
- Samples in TRIzol are stable at room temperature or refrigerated for several hours
- Long term storage of samples in TRIzol should be at -20°C or colder

Prepare sample for RNA extraction

Step 15.

Additional resources

2. Additional recommendations for performing the DFA test can be found at https://www.cdc.gov/rabies/pdf/RabiesDFASPv2.pdf
3. A video describing necropsy procedures is available from the CDC.

References

Step 16.

1. Manning, S.E., et al., *Human rabies prevention--United States, 2008: recommendations of the Advisory Committee on Immunization Practices.* MMWR Recomm Rep, 2008. **57**(RR-3): p. 1-28.

2. World Health Organization, *WHO Expert Consultation on Rabies. Second report.* World Health Organ Tech Rep Ser, 2013(982): p. 1-139. PMID: 16485446
3. World Organization for Animal Health (OIE), *Rabies (infection with rabies virus)* in *Manual of diagnostic tests and vaccines for terrestrial animals*. 2017. http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.01.17_RABIES.pdf

Warnings

Hazards and Safety Precautions

- Samples may contain infectious agent(s). You should be aware of the health hazard presented by such agents and should use, store, and dispose of such samples in accordance with the required safety regulations.
- Wear appropriate personal protective equipment (heavy rubber gloves or other cut-resistant gloves, laboratory gown, waterproof apron, surgical mask, boots, protective sleeves, and a face shield).
- Follow procedures as demonstrated in the Biosafety in Microbiological and Biomedical Laboratories (BMBL), 5th Edition (https://www.cdc.gov/biosafety/publications/bmbl5/bmbl.pdf)
- All manipulations of tissues should be conducted in a manner that does not aerosolize liquids or produce airborne particles.
- Pre-exposure rabies vaccination, regular serologic tests, and booster immunizations as necessary are required for all persons prior to working with lyssaviruses or with known or potentially infected specimen, or engaging in diagnostic, production, or research activities with these viruses [1-3].
- Fume hoods or biosafety hoods are not required but do provide additional protection from odor, ectoparasites, and bone fragments.
- If an electric saw is used to cut through the bone of the skull, then a class II biosafety cabinet is required.
- Some chemicals used with this assay may be hazardous or become hazardous; refer to the SDS as needed. Dispose of chemical waste as directed in the SDS and according to local regulations.
- TRIzol reagent is a hazardous chemical; contact with acids or bleach liberates toxic gases; ensure adequate ventilation; please refer to the safety data sheet for more information.