Rabies virus (RABV) isolation and culture.

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This protocol describes the tissue culture and live animal technique for the amplification of viable rabies virus (RABV) referred to our lab. RABV is a group V ((-)ssRNA) and belong to the Mononegavirales order, Rhabdoviridae family, Lyssavirus genus. These viruses are enveloped and have a single stranded negative-sense RNA genome bound into a helicoidal ribonucleoprotein complex. The genome encodes five genes of conserved order for nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and the viral RNA polymerase (L). The complete genome sequences range from 11,615 to 11,966 nt in length. Upon infection, all transcription and replication events take place in a 2–10 µm cytoplasmic vacuole called the Negri body. Lyssaviruses are characterized by an extremely broad host spectrum ranging from plants to insects and mammals; human-infecting viruses more commonly icosahedral in symmetry and take shapes approximating regular polyhedra. The two different approaches mentioned here allow viable RABV particles to be amplified, isolated and stored for downstream procedures or biobanking. This is done either through a tissue culture technique or by means of intracerebral inoculation of live animals. The in vitro amplification of RABV should be used when the Fluorescent antibody test (FAT) result is uncertain or when the FAT is negative and the case is linked to human exposure. Virus isolation through tissue culture techniques should be preferred over the mouse inoculation test (MIT). Tissue culture amplification of RABV is as sensitive as MIT but less expensive, provide faster results and avoids the use of animals.

In vitro amplification (Tissue culture approach)

1. A couple of days before attempting to amplify virus, grow enough volume of susceptible N2a or BHK-21 cells in a tissue culture flask until 70% confluence. Reference labs should maintain a fresh stock of this cell culture ready for use upon sample referral.

2. Minutes before inoculating plates add 200 µL of a 2×10^5 cells/ml suspension of the cells mentioned in step 1 to four wells of a 96-well plate.

3. Add 100 µL of clarified brain homogenate (20% in phosphate buffered saline, 0.1 M, pH 7.4).

4. Incubate for 24 hours at 5% CO_2 and 37°C.

5. After 24 hrs. harvest supernatant from each well and add 200 µL of fresh medium to each well. Reduce incubation time if cytotoxicity is seen before

6. Incubate for 72 hours at 5% CO_2 and 37°C.

7. After 72 hrs. harvest supernatant from each well and store as viral stock.

8. Fix cells with 80% acetone and stain with fluorescent antibody according to manufacturers' recommendations.

9. Up to three passages may be considered to increase sensitivity.

Notes on the *in vitro* (tissue culture) approach

1. Cell culture tests and their variations should be fully validated before use.
2. Neuroblastoma cells (i.e., N2a) are highly susceptible to infection with lyssaviruses.
3. Cells are grown in Dulbecco's modified Eagle's medium (DMEM) with 5% fetal calf serum (FCS), incubated at 36°C with 5% CO_2.
4. Baby hamster kidney (BHK-21) cells are also sensitive to most street isolates but should be checked for susceptibility to locally predominant virus variants.
5. Cell culture tests should be undertaken in multi-well plastic plates.
6. Use of one 4-day passage in four wells of a 96-well microtitre plate has been shown to have comparable sensitivity to MIT for rabies strains. However, additional passages increase sensitivity.
7. Cytotoxicity normally limits test robustness so use of antibiotics, reducing the time before changing media and dilution of samples can be used to prevent this.

***In vivo* amplification (Mouse inoculation approach)**

1. Three-to-ten mice, 3–4 weeks old (12–14 g), or a litter of 2-day-old newborn mice, are inoculated intracerebrally after being anesthetized.
2. The brain inoculum is prepares as a clarified supernatant of a 10–20% (w/v) homogenate of brain material including brainstem (e.g. cortex, Ammon's horn, thalamus, medulla oblongata) in an isotonic buffered solution containing antibiotics.
3. Alternative inoculums have been prepared using normal saline infected or suspected brain homogenates (10% brain weight to saline volume).
4. Anesthetize rodents in chloroform vapour chamber or intraperitoneal Xylazine (13 mg/kg) + Ketamine (87 mg/kg).
5. Place rodent on ventral decubitus position using an immobilization board fitted with rubber bands. Trace an imaginary line running from the lateral eye cantus to the base of the contra lateral ear. Do the same for the other side mark this spot with permanent ink pen.

Intracerebral inoculation of RABV-infected brain suspension

6. Using an insulin syringe with 26-27 G needle, inoculate 0.03 ml of brain homogenate by piercing through the skull at this marked point. Needle should penetrate cranial cavity between 2 to 3 mm.

7. Mice are to be kept under strict surveillance, preferably in an ABSL2 or BSL3 setting when inoculating with wild animal homogenates (as exotic pathogens might be present), with free access to food and water.

8. The mice are observed daily for 28 days, and dead mice are subjected to necropsy to obtain brain tissue that is explored through either PCR or FAT.

9. Clinical signs indicative of RABV infection may include restlessness, teeth grinding, head tremors, poor coordination of the hind limbs, hackles, ascending paralysis and death.

10. Deaths occurring during the first 4 days are regarded nonspecific (due to stress/bacterial infection etc.).

11. For faster results in newborn mice, it is possible to check one baby mouse by FAT on days 5, 7, 9 and 11 post-inoculation.

Notes on the in vitro (tissue culture) approach

1. Use of specific pathogen free (SPF) rodents is recommended.

2. MIT may also detect viruses other than rabies virus.

3. Mice should be anaesthetized when inoculated.

4. Once a validated and reliable cell culture unit exists in the laboratory, consideration should be given to replacing the MIT with the tissue culture approach as soon as possible as it avoids the use of live animals, is less expensive and gives faster results.

5. A major advantage of MIT is that when the test is positive, a large amount of virus is readily available for downstream procedures such as shipment, characterization, sequencing and typing.

6. MIT can be easily and practicably applied in situations where skills and facilities for other tests (e.g. cell culture) are not available.

References

1. World organization for Animal Health. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2017. Chapter 2.1.17. Accessed from www.oie.int/en/international-standard-setting/terrestrial-manual/access-online/ by july 16, 2017.

2. Rudd RJ, Trimarchi CV. Development and evaluation of an in vitro virus isolation procedure as a replacement for the mouse inoculation test in rabies diagnosis. J Clin Microbiol. 1989 Nov;27(11):2522-8. PubMed PMID: 2681254; PubMed Central PMCID: PMC267070.

Revision history

1.0 Original document.

2.0 Changes to document format only.