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Laboratory standard operating procedure for lyssavirus surveillance of the bat (Chiroptera) population in Taiwan

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TITLE:

Standard Operating Procedure for Lyssavirus Surveillance of the Bat Population in Taiwan

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KEYWORDS:

rabies, lyssavirus, surveillance, bat, Chiroptera, standard operating procedure, Taiwan

SUMMARY:

This protocol introduces a standard laboratory operating procedure for diagnostic testing of lyssavirus antigens in bats in Taiwan.

ABSTRACT:

Viruses within the genus *Lyssavirus* are zoonotic pathogens, and at least seven lyssavirus species are associated with human cases. Because bats are natural reservoirs of most lyssaviruses, a lyssavirus surveillance program of bats has been conducted in Taiwan since 2008 to understand the ecology of these viruses in bats. In this program, non-governmental bat conservation organizations and local animal disease control centers cooperated to collect dead bats or bats dying of weakness or illness. Brain tissues of bats were obtained through necropsy and subjected to direct fluorescent antibody test (FAT) and reverse transcription polymerase chain reaction (RT-PCR) for detection of lyssavirus antigens and nucleic acids. For the FAT, at least two different rabies diagnosis conjugates are recommended. For the RT-PCR, two sets of primers (JW12/N165-146, N113F/N304R) are used to amplify a partial sequence of the lyssavirus nucleoprotein gene. This surveillance program monitors lyssaviruses and other zoonotic agents in bats. Taiwan bat lyssavirus is found in two cases of the Japanese pipistrelle (*Pipistrellus abramus*) in 2016–2017. These findings should inform the public, health professionals, and scientists of the potential risks of contacting bats and other wildlife.

INTRODUCTION:

Viruses within the genus *Lyssavirus* are zoonotic pathogens. There are at least seven lyssavirus species associated with human cases[1]. In addition to the 16 species in this genus[1-3], Taiwan bat lyssavirus (TWBLV)[4] and Kotalahti bat lyssavirus[5] have been recently identified in bats, but their taxonomic statuses have yet to be determined.

Bats are the natural hosts of most lyssaviruses, with the exception of Mokola lyssavirus and Ikoma lyssavirus, which have yet not been identified in any bats[1-3,6]. The information on lyssaviruses in Asian bats is still limited. Two uncharacterized lyssaviruses in Asian bats (one in India and the other in Thailand)[7,8] have been reported. One human rabies case associated with a bat bite in China was reported in 2002, but the diagnosis was made only by clinical observation[9]. In Central Asia, Aravan lyssavirus was identified in the lesser mouse-eared bat (*Myotis blythi*) in Kyrgyzstan in 1991, and Khujand lyssavirus was identified in the whiskered bat (*Myotis mystacinus*) in Tajikistan in 2001[10]. In South Asia, Gannoruwa bat lyssavirus was identified in the Indian flying fox (*Pteropus medius*) in Sri Lanka in 2015[3]. In Southeast Asia, several serological studies on bats in the Philippines, Thailand, Bangladesh, Cambodia, and Vietnam showed variable seroprevalence[11-15]. Although Irkut lyssavirus was identified in the greater tube-nosed bat (*Murina leucogaster*) in Jilin Province, China in 2012[16], the exact species and locations of lyssaviruses in East Asian bat populations remain unknown.

To assess the presence of lyssavirus in Taiwanese bat populations, a surveillance program employing both direct FAT and RT-PCR was initiated. Taiwan bat lyssavirus was identified in two cases of the Japanese pipistrelle (*Pipistrellus abramus*)[4] in 2016–2017. In the present article, a laboratory standard operating procedure is introduced for lyssavirus surveillance of the bat population in Taiwan. The flow chart of bat lyssavirus diagnosis in our laboratory is presented in **Figure 1**.

PROTOCOL:

1. Safety precautions when handling lyssaviruses

1.1. Ensure that all laboratory workers handling bat specimens receive pre-exposure rabies prophylaxis[17]. Monitor the rabies antibody levels of the workers beforehand and re-examine them every 6 months[17]. Follow-up rabies vaccination is required for those whose antibody levels are lower than 0.5 IU/mL[17].

1.2. Depending on the biosafety regulations of the country where the laboratory is located, ensure that the following procedures are performed in suitable biosafety level laboratories (e.g., BSL-2 laboratories in Taiwan and BSL-3 laboratories in Australia), and that workers are currently qualified and wear proper personal protective equipment[18].

NOTE: Rabies vaccination provides little to no protection from lyssaviruses belonging to

phylogroups II and III[18]. Workers must be informed that several zoonotic pathogens have been identified in bats[19] and should handle samples under suitable biosafety level laboratory conditions with proper personal protective equipment.

2. Sample collection

2.1. Use found weak or sick bats or carcasses.

NOTE: Weak or sick bats are delivered to the Bat Conservation Society of Taipei for veterinary care or research, while carcasses are submitted directly to the Animal Health Research Institute. No healthy bats have been euthanized in this surveillance program.

2.2. Have a bat ecologist identify bat species through morphological characteristics by a bat ecologist[20].

2.3. Perform DNA barcoding of the bat species when lyssavirus positive is diagnosed, using previously published procedures[21].

2.4. Submit an information sheet of each bat (collection site, species, clinical signs, etc.) submitted with each bat carcass.

3. Necropsy of bat specimens

3.1. Prepare materials.

3.1.1. Prepare a clean dissection board and place a sterile absorbent pad for necropsy.

3.1.2. Prepare collection tubes for collecting bat organs.

3.1.3. Prepare disposable tweezers and scalpels for necropsy. Change tools between each bat's necropsy. Prepare cotton balls moistened with 70% ethanol for cleaning tweezers and scalpels during sampling.

3.1.4. Prepare two microscope slides for the FAT. Collect fresh specimens and fix them in formalin solution for histopathological examination.

3.2. Examine all external orifices before necropsy. Photograph the bat's external features, especially the head, ears, and wings, for species differentiation.

3.3. Collect an oral swab sample. Place the bat in ventral recumbency on the board and fix the bat's head with tweezers. Cut the skin along the midline of the calvaria with a scalpel and pull the skin to the lateral sides. Cut the skull along the midline of the calvaria with a scalpel and open it with tweezers to expose the brain tissue.

3.4. Remove the brain tissue from the skull and place it on a sterile tongue depressor, and make impression smears from the brain tissue (see step 4.2). Collect a small piece of fresh brain tissue and fix it in formalin for histopathological examination. Contain the remaining brain tissue in a tube for nucleic acid extraction.

3.5. Clean tweezers and scalpel with cotton balls moistened with 70% ethanol to remove retained tissues between specimen collection.

3.6. Place the bat on the board in dorsal recumbency and fix it on the board with needles at both sides of the axilla and tail heel.

3.7. Incise the skin along the midline of the body from mandible to anus. Lift and separate the skin and underlying muscle tissues with tweezers. Collect the salivary glands, which are near the mandibular bone.

3.8. Lift the sternum slightly with tweezers, and cut the sternum and abdominal wall along the midline with a scalpel. Cut the clavicles with a scalpel. Fix the left and right rib cages to the board with needles to open the thoracic cavity.

3.9. Record the gross lesions and the degree of post-mortem change.

3.10. Remove the visceral tissues (i.e., heart, lungs, liver, kidneys, intestines) from the carcass using tweezers and a scalpel. Collect the visceral specimens as needed for future research.

NOTE: Collection of duplicate samples is recommended. One should be collected for molecular diagnosis, and the other should be frozen at -80 °C with or without viral transport medium for viral culture[22].

4. Direct fluorescent antibody test (FAT)

4.1. Make impression smears of the brain tissue for the FAT. Perform the FAT as previously described[18,23] with the following modifications.

4.2. Gently separate the brain tissue from the connected nerve tissue with tweezers and transfer the brain tissue to a sterile tongue depressor. Cut the cross-section of the brain, including the brain stem and cerebellum[18,23]. Make impression smears of the brain tissue by lightly touching the cut surface of the brain tissue, and press the slide on lens tissue to remove excess tissue.

4.3. Fix the slides with acetone at -20 °C for 30 min. Dry the tested slides and the positive and negative controls before staining with conjugate.

4.4. Using each of the two commercially available FITC-conjugated anti-rabies antibodies for

staining lyssavirus antigen is highly recommended[23]. Determine the working concentration of the commercial conjugate before the first staining. Drop the diluted conjugates through a 0.45 µM syringe filter onto the slides and incubate the slides at 37 °C for 30 min within a wet chamber.

4.5. Drain the excess conjugate from the slides and wash the slides with phosphate-buffered saline (PBS) after incubation.

4.6. Drop a small amount of 10% glycerol on the slides and cover with cover slides.

4.7. Examine the slides with a fluorescent microscope.

5. Nucleic acid extraction

5.1. Add the proper volume of MEM-10 (minimum essential medium supplemented with 10% fetal bovine serum) to the brain tissue (10% w/v).

5.2. Homogenate the brain tissue with a 5 mm steel bead in a homogenizer instrument and centrifuge at 825 x *g* for 10 min.

5.3. Extract the nucleic acid, the final volume of which is 50 µL, within 200 µL of the supernatant using the commercially available total nucleic acid extraction kit with instrument.

6. RT-PCR and phylogenetic analysis

NOTE: Several primer sets have been published to detect all known lyssaviruses or specific lyssaviruses. The protocol described here is an example that our laboratory uses and may not fit all experimental needs. Select suitable primers according to laboratory needs.

6.1. Prepare the one-step RT-PCR reagent as follows: add 5 µL of extracted nucleic acid to the reaction mixture containing 2.5 µL of 10x reaction buffer, 0.5 µL of forward and reverse primers (10 µM of each), 4 µL of 1.25 mM dNTP, 0.3 µL of RNase inhibitor (40 U/µL), 0.3 µL of reverse transcriptase (10 U/µL), 0.4 µL of DNA polymerase (5 U/µL), and 11.5 µL of DEPC-treated water.

NOTE: The primer set used in this protocol is JW12 (5'-ATGTAACACCYCTACAATG-3') and N165-146 (5'-GCAGGGTAYTTRTACTCATA-3')[24]. Modify the preparation of reagents and cycling conditions according to the primer sets used.

6.2. Perform the cycling under the following conditions: incubation at 42 °C for 40 min; initial denaturation at 94 °C for 10 min; 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; and finally, further extension at 72 °C for 10 min.

6.3. Use another or more primer sets to increase the diagnostic sensitivity.

6.3.1. Use N113F (5'-GTAGGATGCTATATGGG-3') and N304R (5'-TTGACGAAGATCTTGCTCAT-3')[25-26] to prepare the one-step RT-PCR reagent as follows: add 5 µL of extracted nucleic acid to a reaction mixture containing 5 µL of 10x buffer, 5 µL of forward and reverse primers (4 µM of each), 5 µL of 1.25 mM dNTP, 0.5 µL of RNase inhibitor (40 U/µL), 0.2 µL of reverse transcriptase (10 U/µL), 1 µL of DNA polymerase (5 U/µL), and 23.3 µL of DEPC-treated water.

6.3.2. Perform the cycling under the following conditions: incubation at 42 °C for 40 min; initial denaturation at 95 °C for 5 min; 35 cycles of 95 °C for 1 min, 55 °C for 1 min and 20 s, and 72 °C for 1 min; and finally, further extension at 72 °C for 10 min.

NOTE: Depending on laboratory needs, choose suitable primers for diagnosis. N113F was originally designed for rabies virus amplification, but it may not work well for other lyssaviruses. The set of N113F and N304R works well for rabies virus (Taiwan ferret badger variant) and Taiwan bat lyssavirus. It will be easier to obtain whole nucleoprotein sequences by using the set of JW12 and N304R primers if the lyssavirus is amplified by both of the above two primer sets.

6.4. Analyze the PCR product on 2% agarose gel electrophoresis and visualize by UV light illumination.

6.5. Sequence the PCR product by commercial sequencing service.

6.6. Enter or upload the sequence to the webpage of the Nucleotide Basic Local Alignment Search Tool (BLAST). Select the "others (nr etc.)" database and enter the organism as **Lyssavirus**. Select the MegaBlast algorithm and run the BLAST.

7. Virus isolation

NOTE: Perform virus isolation when either 1) the FAT or 2) the RT-PCR indicates positivity.

7.1. Homogenize the brain specimen in a 10% (w/v) suspension in MEM-10. Centrifuge at 825 x *g* for 10 min.

7.2. Inoculate 200 µL of supernatant with a suspension of 3 x 10^6 MNA (mouse neuroblastoma) cells in 1 mL of MEM-10 for 1 h at 37 °C with 1% CO_2. Transfer the brain homogenate-cell suspension to a 25 cm^2 flask and add 6 mL of MEM-10.

7.3. Cultivate 1 mL of the brain homogenate-cell suspension in a 4 well Teflon-coated glass slide with 6 mm diameter at the same time.

7.4. After 3–4 days of incubation at 37 °C with 1% CO_2, fix the cells on the 4 well slide with 100% acetone (v/v).

7.5. Stain the slides with two FITC-conjugated anti-rabies antibodies following steps 4.4–4.7. The cells are infected when the intracytoplasmic inclusions are investigated. Record the percentage of infected cells.

7.6. Perform trypsinization and subculture of the inoculated cell culture when the slides are stained as negative:

7.6.1. Remove medium and rinse the flask with 5 mL of PBS.

7.6.2. Add 1 mL of trypsin to the flask and firmly strike the bottom of the flask.

7.6.3. Add 6 mL of MEM-10 and resuspend the cells.

7.6.4. Put the cell suspension into a new tissue flask (6 mL) and onto a 4 well slide (1 mL).

7.7. Repeat steps 7.4–7.6 until 100% infectivity is reached.

7.8. Collect the supernatant after 24 h of incubation.

REPRESENTATIVE RESULTS:

From 2014 to May 2017, 332 bat carcasses from 13 species were collected for surveillance. Two tested positive. In the first bat case, the brain impression tested negative using the FAT with one of the commercial FITC-conjugated anti-rabies antibodies (**Figure 2**), while the RT-PCRs employing each of the two primer sets (JW12/N165-146, N113F/N304R) yielded positive results (**Figure 3**). A 428 bp sequence of amplicon (amplified with N113F/N304R and containing the partial nucleoprotein gene) was obtained. Its sequence was subjected to BLAST querying by the GenBank database. The result showed that the sequence was similar to lyssaviruses with identities of less than 79% (**Figure 4**), supporting the identities of the detected lyssaviruses.

Later, two lyssaviruses were isolated successfully from these two brains, and the viruses were confirmed by FAT (**Figure 5**) and sequencing. The identified lyssavirus was designated as Taiwan bat lyssavirus (TWBLV) based on the sequence analysis[4]. In the second case, the results obtained from the FATs employing each of the two commercial FITC-conjugated anti-rabies antibodies were inconsistent, as described for the first case.

FIGURE & TABLE LEGENDS:

Figure 1: Bat lyssavirus diagnosis flow chart. Flowchart showing the current process and diagnostic methods which our laboratory used now. Virus isolation should be performed when either the direct fluorescent antibody test or the reverse transcription polymerase chain

reaction is positive.

Figure 2: Direct FAT with two commercial FITC-conjugated anti-rabies antibodies of whole brain compression from a TWBLV-infected bat yielding inconsistent results. Case number: 2016-2300: (A) The FAT with Reagent A (5x dilution), demonstrating apple green positive signals. (B) The FAT with Reagent B, showing a negative result (20x dilution).

Figure 3: Products of RT-PCRs employing two primer sets. The primer set used in lanes 1 to 3 was JW12/N165-146, and the expected product size was 111 base pairs. The primer set used in lanes 4 to 6 was N113F /N304R, and the expected product size was 521 base pairs. Both tests of the sample (lanes 1 and 4) were positive. M = 100 bp DNA ladder; lanes 1 and 4 = tested sample; lanes 2 and 5 = positive controls; lanes 3 and 6 = negative controls.

Figure 4: BLAST result of N113F/N304R product of TWBLV infected bat. BLAST results showed that the case was most similar to the lyssavirus, but the identity with the lyssavirus in the database was only 79%.

Figure 5: Comparison of lyssavirus antigen distribution of virus isolation of TWBLV with two FITC-conjugated anti-rabies conjugates. The 10% bat brain emulsion (TWBLV infected) was inoculated into mouse neuroblastoma cells for virus isolation. The FATs were performed at the tenth passage and stained with two FITC-conjugated anti-rabies antibodies. Antigen distribution of mouse neuroblastoma cells with two rabies conjugates showed significant differences. (A) The FAT with Reagent A (5x dilution). (B) The FAT with Reagent B (5x dilution).

DISCUSSION:

This laboratory standard operating procedure (SOP) provides a serial process for testing bat samples for the presence of lyssavirus antigens in Taiwan. The key steps include the employment of FAT and RT-PCR. The selection of suitable samples and successful isolation of the virus are also important. Additionally, some troubleshooting was conducted during the monitoring of bat lyssaviruses. The major difference was the target animals. Initially (2008–2009), the target animals of bat lyssavirus surveillance were live bats, which were trapped in Kinmen Island in Taiwan then tested for lyssavirus after euthanasia. Most of the trapped bats were healthy and unlikely to carry lyssavirus, and this approach to monitoring was not humane. Therefore, in the third year, only dead or dying bats were collected and expand the surveillance area from regional to national. After eight years of continuous monitoring, the first bat lyssavirus case was finally detected in Taiwan.

Although FAT is the most widely used method for rabies diagnosis and recommended by OIE and WHO[18], few studies have demonstrated inconsistent results when different conjugates were used in the FAT[5,27]. Similar inconsistent results also appeared in TWBLV-infected cases. In the TWBLV-infected MNA cells, the results of FAT showed significant differences in 2 conjugates (**Figure 5**). One of the FITC-conjugated anti-rabies antibodies did not react well, even at higher concentrations. Because of the variation of lyssavirus antigen in the samples and the variation

of the antibody avidity and affinity of antibodies in the conjugates, it is recommended that two different conjugates be used in FAT to prevent false negative results in lyssavirus diagnosis[23,28,29].

RT-PCR can provide confirmatory diagnosis for inconsistent results of FAT. Due to the high genetic diversity of lyssavirus, it is recommended that more than one primer set in RT-PCR be used to increase the accuracy of lyssavirus screening[29,30]. A primer set designed from highly conserved nucleoprotein genes is the most commonly used set in lyssavirus detection[29]. RT-PCR can also be used for diagnosis in putrefactive samples when FAT cannot be performed[31,32]. To avoid false negativity preventing the discovery of a novel lyssavirus, more tools are recommended for detection. Two novel lyssaviruses[4], Taiwan bat lyssavirus, were identified during this survey employing the SOP.

Additionally, one highly diverse TWBLV strain and a novel new species of lyssavirus were found in bats in Taiwan in 2018 (unpublished data). The findings proved that employment of both the FAT and RT-PCR to detect lyssaviruses in bats is useful. Some limitations of the RT-PCR primer set used in this SOP should be noted. In the primer set of N113F/N304R, N113F was originally designed for rabies virus amplification, but it may not work well for other lyssaviruses. Several primers for lyssavirus detection have been published by other researchers[29-30] and can be chosen according to laboratory needs.

This article is a step-by-step introduction to bat lyssavirus surveillance in Taiwan. It is hoped that this SOP will be helpful to researchers who are interested in bat lyssavirus surveillance. As more researchers carry out surveys of bats, more lyssaviruses will be identified in the future. This SOP monitors not only lyssaviruses but also other zoonoses agents in bats. Such findings can inform the public, health professionals, and scientists of the potential risks of contact with bats and other wildlife. It will also help increase understanding of the evolution and origins of the lyssavirus and lead to substantial progress in scientific research.

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DISCLOSURES:

No conflicts of interest are declared.

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Figure 1

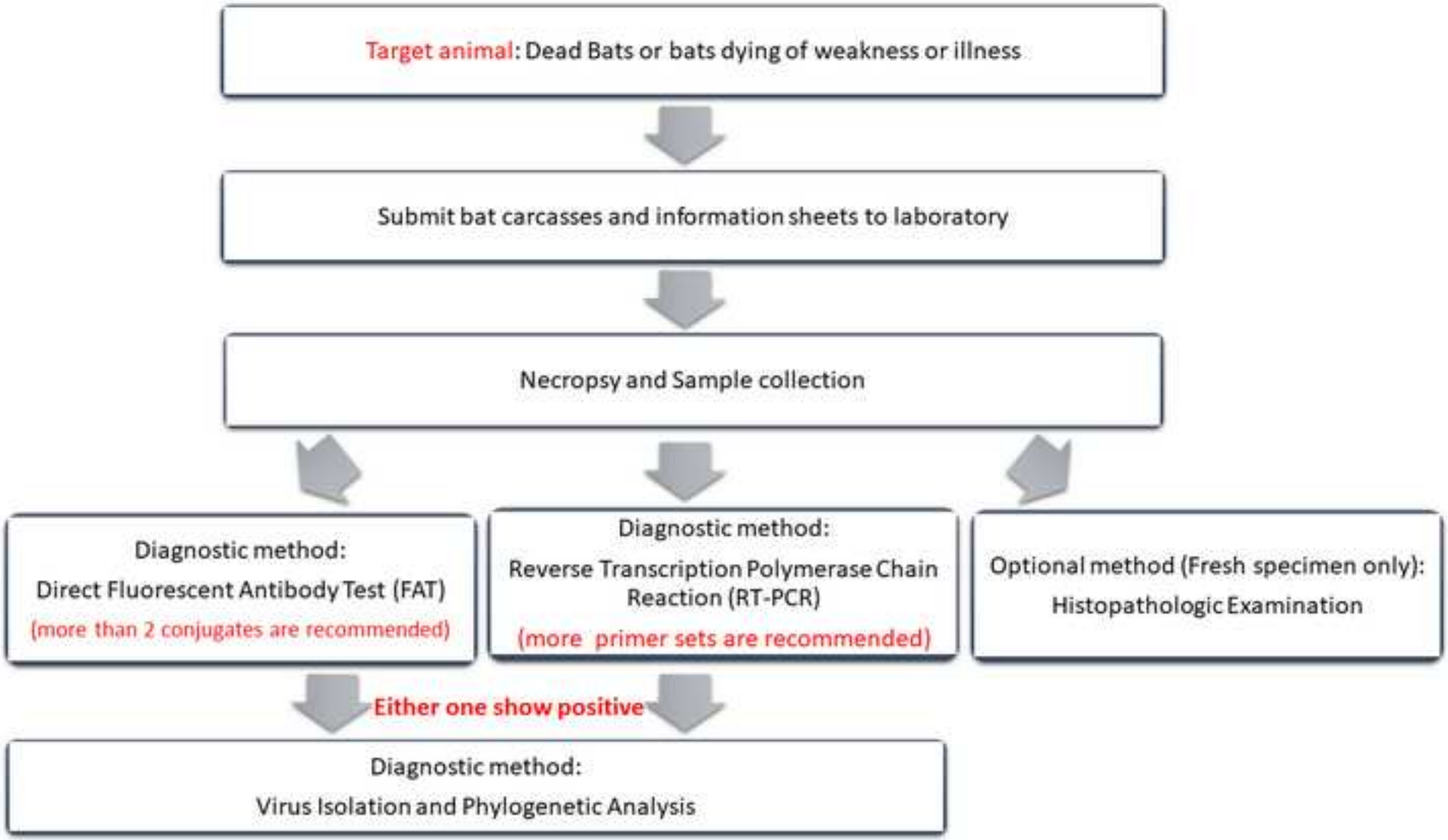

Figure 2

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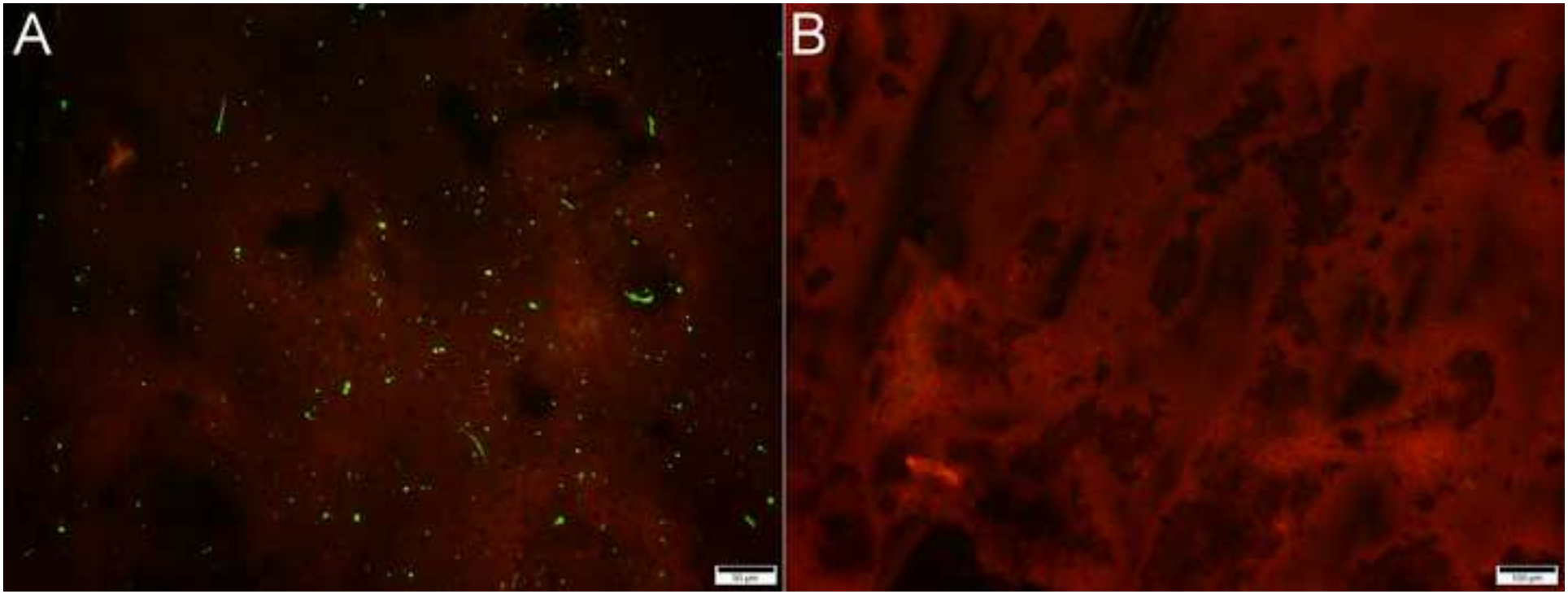

Figure 3

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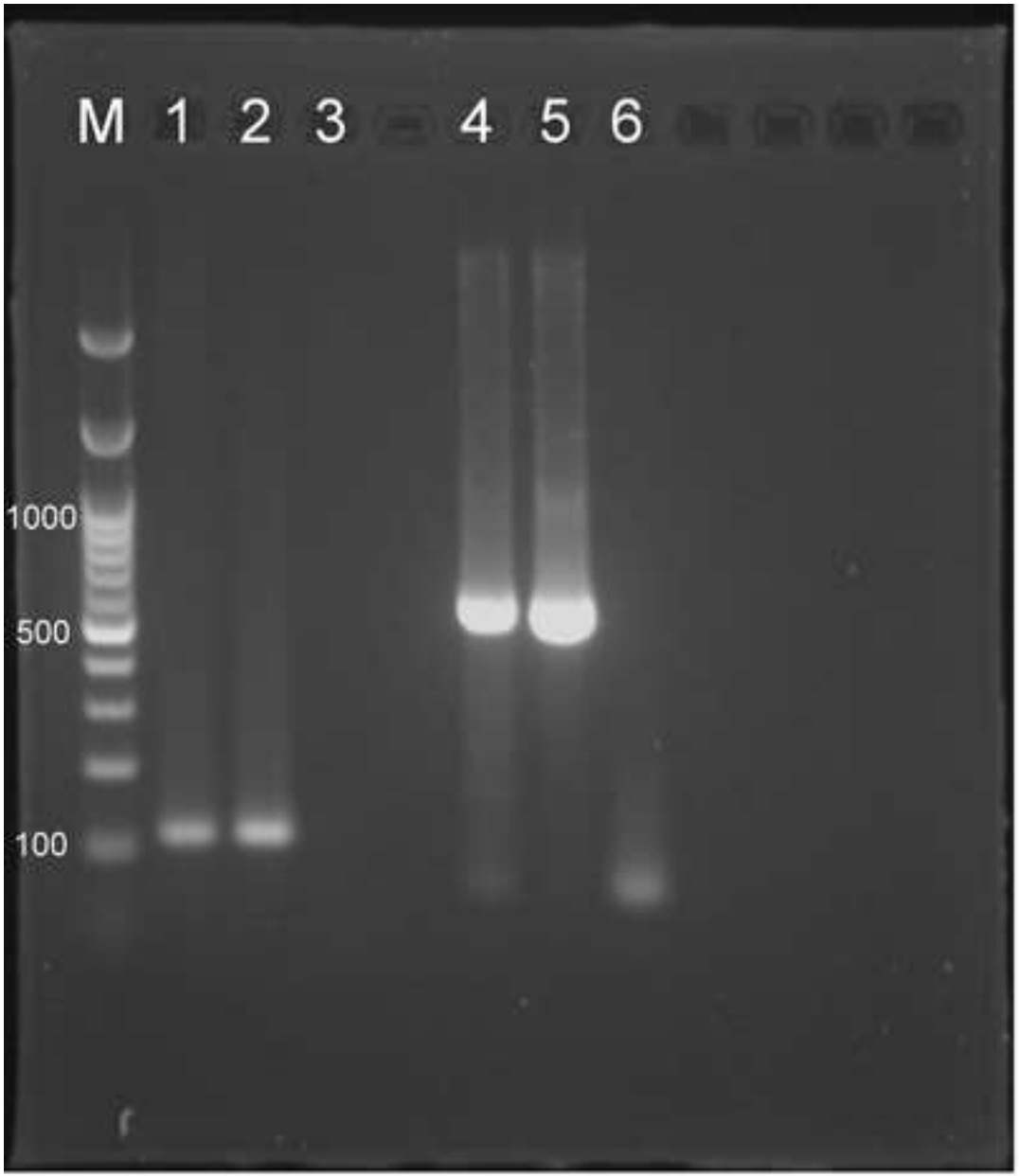

Figure 4

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Molecule type nucleic acid
Query Length 428

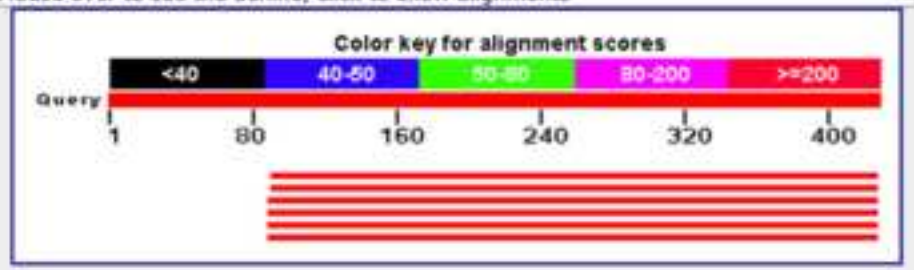
Database Name nr
Description Nucleotide collection (nt)
Program BLASTN 2.5.0+ ▶Citation

Other reports: ▶Search Summary [Taxonomy reports] [Distance tree of results]

Graphic Summary

Distribution of 6 Blast Hits on the Query Sequence

Mouse over to see the defline, click to show alignments



Descriptions

Sequences producing significant alignments:

Select: All None Selected 0

Alignments

Description	Max score	Total score	Query cover	E value	Ident	Accession
Lagos bat virus isolate KE578, complete genome	233	233	78%	2e-57	79%	GU170202.1
Lagos bat virus isolate KE478, complete genome	228	228	78%	9e-56	79%	JX901139.1
Rabies virus isolate M839 nucleoprotein (N) gene, partial cds	220	220	78%	2e-53	78%	GU644820.1
Rabies virus isolate M399 nucleoprotein (N) gene, complete cds	220	220	78%	2e-53	78%	GU644669.1
Rabies virus isolate M782 nucleoprotein (N) gene, complete cds	220	220	78%	2e-53	78%	GU644663.1
Rabies virus isolate VA1623 nucleoprotein (N) gene, complete cds	215	215	78%	7e-52	78%	GU644678.1

Figure 5

Click here to access/download;Figure;Fig - 4_upload.tif

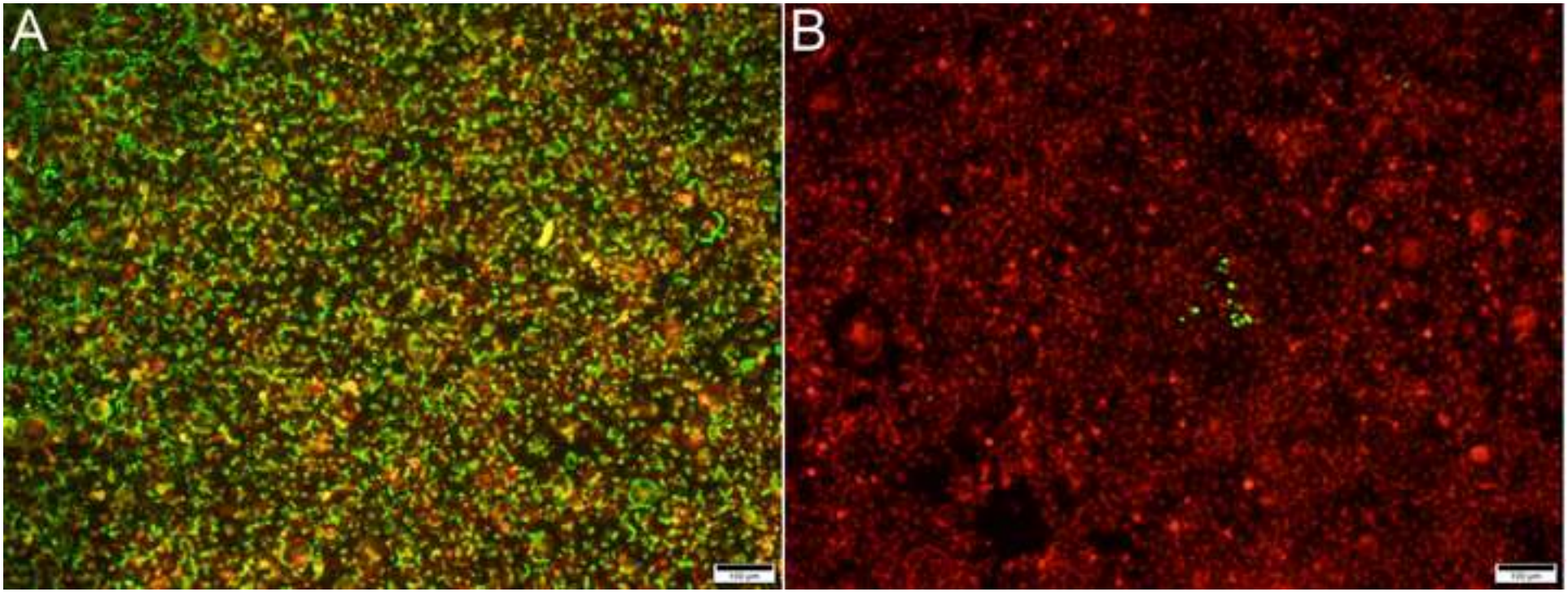


Table of Materials

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
2.5% Trypsin (10x)	Gibco	15090046	Trppsin
25 cm^2 flask	Greiner bio-one	690160	
Acetone	Honeywell	32201-1L	
Agarose I	VWR Life Science	97062-250	
Alcohol	NIHON SHIYAKU REAGENT	NS-32294	
AMV Reverse Transcriptase	Promega	M5101	
Antibiotic-Antimycotic(100X)	Gibco	15240-062	MEM-10
Blade	Braun	BA215	
Centrifuge	eppendorf	5424R	
Chemilumineance system	TOP BIO CO.	MGIS-21-C2-1M	
Collection tube	Qiagen	990381	
Collection tube	SSI	2341-SO	
Cover slide	Muto Pure chemical Co., LTD.	24505	
DNA analyzer	Applied Biosystems	3700XL	
Fetal bovine serum	Gibco	10437028	MEM-10
FITC Anti-Rabies Monoclonal Globulin	Fujirebio Diagnostic Inc.	800-092	FITC-conjugated anti-rabies antibodies: reagent B
Four-well Teflon-coating glass slide	Thermo Fisher Scientific	30-86H-WHITE	
Gel Electrophoresis System	Major Science	MJ-105-R	
HBSS (1x)	Gibco	14175095	Trppsin
Incubator	ASTEC	SCA-165DS	
Inverted Microscope	Olympus	IX71	
L-Glutamine 200 mM (100x)	Gibco	A2916801	MEM-10
LIGHT DIAGNOSTICS Rabies FAT reagent	EMD Millipore Corporation	5100	FITC-conjugated anti-rabies antibodies: reagent A
MagNA Pure Compact Instrument	Roche	03731146001	
MagNA Pure Compact NA Isolation Kit 1	Roche	03730964001	
MEM (10x)	Gibco	11430030	MEM-10
MEM NEAA (100x)	Gibco	11140050	MEM-10
MEM vitamin solution	Gibco	11120052	MEM-10
NaHCO3	Merck	1.06329.0500	MEM-10
Needle	Terumo	NN*2332R9	
PBS	Medicago	09-8912-100	
Primer synthesis	Mission Biotech		
RNasin ribonuclease inhibitor	Promega	N2111	
Sequencing service	Mission Biotech		
Slide	Thermo Scientific	AA00008032E00MNT10	
Sodium Pyruvate (100 mM)	Gibco	11360070	MEM-10
Stainless Steel Beads	QIAGEN	69989	
Sterile absorbent pad	3M	1604T-2	
Syringe filter	Nalgene	171-0045	
Taq polymerase	JMR Holdings	JMR-801	
Thermal cycler	Applied Biosystems	2720	
TissueLyser II	QIAGEN	85300	
Tongue depressor	HONJER CO., LTD.	122246	
Tweezer	Tennyson medical Instrument developing CO., LTD.	A0601	
Tylosin Tartrate	Sigma	T6271-10G	MEM-10

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Title of Article: Laboratory Standard operating procedure for lyssavirus surveillance of the bat (chiroptera) population in Taiwan

Author(s): Wei-Cheng Hsu, Chao-Lung Hsu, Yang-Chang Tu, Jen-Chieh Chang, Kwok-Rong Tsai, Fan Lee, Shu-Chia Hu

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CORRESPONDING AUTHOR

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Date:	2019/6/5

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June 5, 2019
Journal of Visualised Experiments

Dear Editor:

On the behalf of my co-authors Wei-Cheng Hsu, Chao-Lung Hsu, Yang-Chang Tu, Jen-Chieh Chang, Kwok-Rong Tsai, Fan Lee and myself, I hereby submit a manuscript entitled “Laboratory standard operating procedure for lyssavirus surveillance of the bat (Chiroptera) population in Taiwan” to the Journal of Visualised Experiments for possible publication.

Thanks for editor's and reviewer’s comments for previous submission and give us the opportunity to optimize our protocol. In the revised manuscript, we edited the manuscript according comments from editor and reviewers. The responses of the editorial and peer review comments are addressed as below. We would be happy to make further corrections if necessary and look forward to hearing from you soon

Thank you very much for considering this manuscript.

Warm Regards,

Shu-Chia Hu

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Editorial comments:

1. Do you have examples of the appropriate safety levels (e.g., in Taiwan or the US)?

Response:Thanks for your comments, the examples of safety level of laboratory in Taiwan and Australia were added in the manuscript.

2. 3.1.3: What is the alcohol? Ethanol?

Response:Thanks for your comments, we replace alcohol with ethanol in the manuscript.

3. 3.4: Please include more information about making impression smears. Also, around how large should the brain tissue sample be?

Response: The information of making impression smears were added step 4.2 (Line 164-168). The size of brain tissue is varied, depended on bat species and size. In Taiwan, the sizes of the collected bats are smaller (body weight varied from 2 to 40 g). The collected brain tissue should be as large as possible and must including the cerebrum, cerebellum, and brain stem.

4. 4.2: Is this the same step as 3.4?

Response: Step 3.4 described the removing step of brain tissue (Line 130-133). Step 4.2 descript the details of making impression smears (Line 164-168).

5. 6.1: 5.3 mentions isolating total nucleic acid, but you only mention RNA here-how is the RNA obtained? Around how much RNA material (i.e., mass) in total should be used?

Response: Thanks for your comments, we replace RNA with nucleic acid in the manuscript (Line 202-203, 219-220). Total 50 μL nucleic acid were extracted from 200 μL of the brain supernatant (10% w/v), and 5 μL nucleic acid were used in the RT-PCR (Line 193, 202, 219).

6. 7.3: What does ‘the same time each passage’ mean?

Response: It means when you passage cells to the flask, you have to prepare a 4-wells slide and also add cells on it. It is much easier to perform FAT on 4-wells slide than on the flask. We deleted “each passage” to make this sentence more clear (Line 258).

7. 7.5: Please include more information here.

Response: Thanks for your comment. We added more detail in step 7.5 (Line 263-265).

Reviewers' comments:

Editor's note: Reviewer 1 has essentially the same comments as before, but their concerns still seem to be largely unsupported by peer-reviewed literature. I think it's fine to essentially overlook comments about whether these are indeed pan-lyssavirus primers, although it might be best to include qualifying statements.

Response: Thanks for your comment. We will test the primer sets which reviewer 1 suggested in the collected samples in the future. However, it is hard to replace our primers with the suggested primer sets in this manuscription due to the insufficient data we have now. To prevent the misleading for detection of diverse lyssaviruses, a note of primer selection was added in Line 198-200.

Reviewer #1:
I disagree with the response given by authors to one of my major comments. Given my experience with PCR-amplification of genes of the majority of lyssaviruses described to date (and all Lagos bat virus lineages), complete genomes and hundreds of primer sets used for diagnostic purposes, I still strongly disagree with author's recommendation on primers used in RT-PCR diagnosis. Indeed, Hayman et al (2011) did not have sufficient experience when described those primers as "pan-lyssavirus". As for negative results of Lagos bat virus amplification which authors want me to refer, simply, such negative records are trivial and do not deserve publication, that's why we and others did not publish them, and I cannot provide a reference requested by authors. Instead, I referred the paper of Markotter et al (2006) where different primers were used, and the reason for using those different primers was exactly that JW12 and N304F did not amplify certain Lagos bat virus lineages. In contrast, primers published by Markotter et al (2006) really amplify N gene of all lyssaviruses described to date. As for the primer N113F, there are so many routine examples when it did not work with various lyssaviruses (including several lineages of rabies virus) that it is a shame that authors continue to stand on the correctness of their method just because of insufficient experience of working with different viruses (as they mentioned, the only two viruses they had in hands were the Chinese ferret-badger variant of rabies virus and Taiwan bat lyssaviruses). Make an alignment and see how poorly the N113F will be aligned with many lyssavirus N genes. As for the approach of using the set JW12:N304R for diagnostic purposes, even in those cases where both primers can be annealed to viral sequence, it is misleading because of the length of amplified product (~1400 bp), which is way too long for diagnostic purposes when high sensitivity is needed (e.g. samples with low virus load, samples with deteriorated viral RNA), and short genome fragments need to be amplified, ideally in nested or hemi-nested reactions.
Response: Thanks for your comment. We will test the primer sets which you suggested in the collected samples in the future. To prevent the misleading for detection of diverse lyssaviruses, a note of primer selection was added in Line 198-200.

The primer set JW12/N304R is not for diagnostic purpose for routine diagnosis, but it will be easier to obtain whole nucleoprotein sequences for further sequence analysis by using the set of JW12 and N304R primers if the lyssavirus is amplified by the two primer sets (N113F/N304R, JW12/N165-146) (Line 233-234).

Therefore, my verdict is the same: authors are not proficient in molecular diagnosis of lyssaviruses (except those 2-3 viruses that they have hands-on experience with), and therefore publication of this method for detection of diverse lyssaviruses (which may be encountered, in particular, in bats) is misleading.

Reviewer #2:
Manuscript Summary:
This report is a success case of virus isolation and very valuable for establishing of bat surveillance for monitoring of lyssaviruses in Asia countries.
Especially, the criteria and decision tree is important for sample collection of bat, biosafety of protocols and PPE in collecting sample and necropsy, and plural tests for conclusion of infection.

Major Concerns:
PROTOCOL: It is more useful if the authors describe at the front about the flow of decisions according to criteria.
Response: The bat lyssavirus diagnosis flow chart (Figure 1) was added in the manuscript.
DISCUSSION: It is of advantage for the monitoring of bat lyssaviruses to know any troubleshooting and modification of protocol until finding first case.
Response: Thanks for your comments, the troubleshooting and modification of protocol were added in the discussion (Line 329-337).

Reviewer #3:
The authors did improve the manuscript and provided more detail on the methodology. There was no rebuttal letter to indicate responses to reviewer comments raised. The following comments raised was not addressed. Line numbers correspond to the original manuscript submitted.

Line 82: Morphological identification of bat species should be followed up with DNA barcoding methods. This should be included in the SOP.

Response: Thanks for your comment. Bat species are identified thorugh morphological characteristics by a bat ecologist, and the additional DNA barcoding will be performed when lyssavirus positive is diagnosed. DNA barcoding is not a routine process in our laboratory. We thought it's will make the SOP too lengthy if the protocol of DNA barcoding was added, so the reference of DNA barcoding was added in the manuscript instead (Line 99-100, reference 21).

Line 93- Distilled water and ethanol will not be sufficient to clean sharps between necropsies. New sharps should be used and this should be sterilized before and after using for example autoclaving or extensive heat. Ethanol will also not destroy nucleic acids present and this will lead to contamination in subsequent PCR reactions.

Response: Sorry for the confusion. The disposable tweezers and scalpel are used for necropsy and change new tools between each bat's necropsy. The manuscript was revised (Line 115-117).

Line104: Tissues are collected in formalin but there is no explanation of why and how this tissue is tested.

Response: The fresh samples are collected and fixed in formalin for histological examination. The manuscript was revised (Line 119-120)

Line 144-148: The dilution of conjugate used is not indicated and it is not indicated which conjugate did not provide results? Why use both if it was clear that the one perform better than the other one?

Response: The working dilution of conjugate should be test before first staining. The working dilution may be different in different conjugates. There are several commercial conjugates can be chosen on laboratory demand. The conjugates which we used was listed in the Table of Material. Different conjugates have different antibody avidity and affinity, it is recommmed to use two differnt conjugates in FAT (reference 23, 28, 29). The conjugate dilution of each figure is added in the manuscript.

Figure 4: The conjugate A and B must be specified. Why would B still show fluorescence in one area and not in the rest. It will bind or not bind. These results does not make sense.

Response: The information of conjugates is listed in the Table of Material. Figure 5B is the result of FAT stained by high working concentration of conjugate B (5x dilution, normal working concentration is 20x in our laboratory). The possible reasons of the difference in FAT between conjugate A and B may be the variation of lyssavirus antigen in samples and the variation of the antibody avidity and affinity of antibody in conjugate.

The manuscript needs substantial editing and grammar improvement

Response: Thanks for your comment. The manuscript was edited again.