Seminar and Laboratory Training on Rabies
Veterinary Research Institute (VRI), Ipoh, Malaysia
7-11 October 2019
Summary

For this training, the OIE RRAP was supported by a team from the OIE Reference Laboratory for Rabies, the Changchun Veterinary Research Institute (CVRI) laboratory in China, led by Dr Changchun Tu. Dr Marzuki bin Zakaria, Deputy Director of the Department of Veterinary Services (DVS) Malaysia, kindly opened the training.

The objective of this course was to provide up-to-date information on the rabies situation both globally and regionally to a variety of stakeholders. During the seminar on the first day, key information on ongoing rabies outbreaks in Malaysia was presented and actively discussed by both veterinary and human health staff. The laboratory training course was also a means to strengthen diagnostic capacity in Malaysia for rabies diagnosis, thereby strengthening surveillance and control activities of the disease. Participants were trained in sampling and preparation of tissues for diagnostic analysis, PCR-based methods for detection of rabies virus RNA and data analysis. The proficiency of the VRI laboratory's fluorescent antibody test (FAT) methodology was evaluated and shown to fully comply with the OIE standard, being conducted by well-trained and highly proficient staff. A great benefit of the visit by OIE rabies expert, Dr Tu, was the opportunity for recommendations to improve the rabies laboratory capacity performance. Dr Tu was able to offer advice on streamlining of the laboratory workflow and optimization of processes for the detection of rabies.

For the seminar on the first day, 63 participants attended; 15 participants from laboratories across the country undertook the hands-on laboratory training. This core group of participants demonstrated increased knowledge of rabies diagnostics over the course of the week and learned several laboratory techniques to strengthen capacity within Malaysia for rabies diagnosis. Dr Tu was helpful in making suggestions to improve laboratory efficiency and safety. After the training, it is hoped that collaboration between CVRI and VRI will continue, including sequence analysis of Malaysian rabies isolates which will be useful in understanding epidemiology of the disease in this country.

**Draft program for the Seminar and Laboratory Training on Rabies
VRI, Ipoh, Malaysia, 7-11 October 2019**

Day 1 (Monday 7 October 2019)

Time	Session 1: <u>Seminar on rabies</u>	Speaker
08:30 – 09:00	Registration of participants	
09:00 – 09:15	Opening remarks	*Deputy Director General of DVS Malaysia Dr. Marzuki bin Zakaria*
09:15 – 09:30	An introduction to the OIE and rabies	*Dr. Lesa Thompson (OIE)*
09:30 – 09:50	Current situation of rabies in animals in Malaysia	*Dr. Sarah Dadang Abdullah (DVS)*
09:50 – 10:20	**Group photograph and coffee break**	*Shift*
10:20 – 10:40	Tripartite activities for control of rabies in Asia & the Pacific	*Dr. Lesa Thompson (OIE)*
10:40 – 11:10	Global and regional WHO updates on rabies elimination	*Dr. Aya Yajima (WHO) presented by Dr. Lesa Thompson (OIE)*
11:10 – 11:40	OIE standards on rabies diagnosis: overview on the rabies chapter of OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals.	*Prof. Changchun Tu (CVRI - OIE Reference Laboratory)*
11:40 – 12:20	Panel discussion on the way forward to strengthen surveillance, prevention and control of rabies in Malaysia.	*Moderator: Prof. Dr.Latiffah Hasan (UPM) Panel: Dr. Lesa Thompson Prof. Changchun Tu Dr. Sarah Dadang Abdullah Dr. Andrea Lim Li Li (DVS Sarawak)*
12:20 – 12:35	Conclusion and summary of the key outcomes	*Dr. Lesa Thompson (OIE)*
12:35 – 12:40	Token of appreciation	*VRI*
12:50 – 14:00	**Lunch**	
Time	Session 2: <u>Laboratory training on rabies</u>	Speaker
14:00 – 14:30	**Lecture:** Introduction of the principles of PCR and real-time PCR	*CVRI - OIE RL*
14:30 – 17:00	**Practical Session I** Hands-on training on sample preparation and	*CVRI - OIE RL*

extraction methods
1. Sample preparation
2. RNA extraction
3. Primer and probe preparation

Day 2 (Tuesday 8 October 2019)

Time	Session 2 /cont.	Speaker
08:30 – 12:00	**Practical Session II** Hands-on training on conventional and real-time PCR (qPCR) and analysis of qPCR result. • qPCR (VRI method) • RT-nPCR (Step 1): cDNA synthesis (OIE RL method)	*CVRI - OIE RL*
12:00 – 13:00	**Lunch**	
13:00 – 17:00	**Practical Session III** Hands-on training on conventional PCR and qPCR • Analysis of qPCR results (VRI method) • RT-nPCR (Step 2): 1st round PCR (OIE RL method)	*CVRI - OIE RL*

Day 3 (Wednesday 9 October 2019)

Time	Session 2 /cont.	Speaker
08:30 – 12:00	**Practical Session IV** Hands-on training for conventional PCR and analysis of PCR results • RT-nPCR (Step 3): 2nd round PCR (OIE RL method)	*CVRI - OIE RL*
12:00 – 13:00	**Lunch**	
13:00 – 17:00	**Practical Session V** Hands-on training for conventional PCR and analysis of PCR results • RT-nPCR (Step 4): gel electrophoresis • Analysis of RT-nPCR results	*CVRI - OIE RL*

Day 4 (Thursday 10 October 2019)

Time	Session 2 /cont.	Speaker
08:30 – 11:00	**Practical Session VI** Demonstration of VRI's operation on dFAT • Preparation of brain tissue smears and fixation for dFAT • Staining and observation	*DVS*
12:00 – 13:00	**Lunch**	
13:00 – 15:00	Assessment of VRI's practice on dFAT	*CVRI - OIE RL*
15:00 – 17:00	**Practical Session VII** Hands-on training on sample preparation Application of FTA cards	*CVRI - OIE RL*

Day 5 (Friday 11 October 2019)

Time	Session 2 /cont.	Speaker
08:30 – 10:15	*Open discussion and practical recommendations* • Laboratory setup • Instruments, software and reagents • Biosafety • QC and QA	*CVRI - OIE RL*
10:15 – 10:30	**Coffee Break**	
10:30 – 11:00	Knowledge quiz Workshop evaluation forms	*DVS/ CVRI - OIE RL*
11:00 – 11:30	Receive workshop completion certificates Concluding remarks Closing ceremony	*DVS/ CVRI - OIE RL* *Prof. Changchun Tu (CVRI - OIE RL)* *Dr. Akma Ngah Hamid (DVS)*
11:30 – 14:00	**Lunch and Friday Prayers**	

LABORATORY TRAINING ON RABIES
Ipoh, Malaysia
October 7-11, 2019

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Introduction

Rabies is caused by neurotropic viruses of the genus *Lyssavirus* in the family *Rhabdoviridae* and is transmissible to all mammals. As the viruses are transmissible to humans, all suspect infected human material must be handled under the appropriate safety conditions specified by the World Health Organization. Laboratories working with lyssaviruses or suspect animal material must comply with national biocontainment and biosafety regulations and as well as following appropriate biosafety and containment procedures as determined by biorisk analysis.

Based on nucleotide sequences of the nucleoprotein gene (N), the lyssairuses are divided into 16 species by ICTV 2018, comprising classical rabies virus (RABV) and the rabies-related viruses (RRV): Lagos bat virus (LBV), Mokola virus (MOKV), Duvenhage virus (DUVV), European bat lyssavirus 1 (EBLV-1), European bat lyssavirus 2 (EBLV-2), Australian bat lyssavirus (ABLV), Irkut virus (IRKV), West Caucasian bat virus (WCBV), Khujand virus (KHUV), Aravan virus (ARAV), Shimoni bat virus (SHIBV), Bokeloh bat lyssavirus (BBLV) and Ikoma virus (IKOV), Lleida bat lyssavirus (LLEBV) and Gannoruwa bat lyssavirus (GBLV). Recently, two novel lyssavirus species were identified as tentative species – Kotalahti bat lyssavirus (KBLV) isolated from a Brandt's bat (Myotis brandtii) in Finland in 2017 and Taiwan bat lyssavirus (TWBLV) isolated from a Japanese pipistrelle (Pipistrellus abramus) in Taiwan, China in 2016-2017.

RABV is found worldwide and is responsible for the overwhelming majority of reported animal and human rabies cases. Other lyssaviruses appear to have more restricted geographical and host range, with the majority having been isolated from bats with limited public and animal health implications. However, all lyssaviruses tested cause clinical disease indistinguishable from RABV.

The lyssaviruses have been divided into at least three phylogroups with distinct pathogenicity and immunogenicity. For phylogroup 1 (comprising RABV, ABLV, EBLV1, EBLV2, BBLV, ARAV, DUVV, KHUV, IRKV, and GBLV), conserved antigenic sites on the surface glycoproteins allow cross-neutralisation and cross-protective immunity to be elicited by rabies vaccination. Rabies virus vaccines, however, may not provide adequate cross-protection against all genetically divergent lyssaviruses. Little or no cross-protection with pre-exposure vaccination and with conventional rabies post-exposure prophylaxis was observed against lyssaviruses of phylogroups 2 and 3.

Purpose/scope of this manual

The diagnoses methods in this document are written according to the OIE "Manual of Diagnostic Tests and Vaccines for Terrestrial Animals" (2018) and the WHO "Laboratory Techniques in Rabies" (Fifth Edition, 2018).

The rabies diagnosis chain

The chain of rabies diagnosis starts in the field with the collection of specimens and ends with a report to the sender. The positive and negative results obtained in the laboratory should then be published in "rabies bulletins" and integrated into national and international statistics.

The steps of this process are therefore:

1. Collection of specimens and epidemiological data in the field

2. Transport of the specimen to the laboratory

3. Choice of test(s)

4. Laboratory diagnosis

5. Disease analysed and reported

Safety

■ Any staff performing this procedure in a rabies high security unit must first be vaccinated, with a demonstrated titre of 1.0 IU/ml or greater.

■ Apparatus (tabletops, socket forceps, scissors, forceps and grinder, etc.) used in the post-mortem room must be cleaned between specimens.

■ A sterile set of instruments should be used for each diagnosis and contaminated instruments should be decontaminated by boiling, autoclaving or adapted chemical treatment.

■ All biological waste material should be decontaminated by incineration. Where this is not possible the waste may be buried in quicklime in an isolated place and at a depth that cannot be reached by carnivores.

■ Potentially infectious material such as brain smears should be fixed before removal from the post-mortem room.

1. Collection of specimens and epidemiological data

Reliable diagnosis of rabies can only be performed from the brain in a laboratory.

Of the parts of the brain, the brainstem is the most reliable for the detection of antigen, followed by the thalamus and hippocampus. Other tissues can also be used for rabies diagnosis, although not all are as reliable as brainstem. These include: spinal cord, peripheral nerve tracts and salivary glands.

Whatever the specimen collected, it must be stressed that brain tissue is required for a reliable rabies diagnosis.

Which information should be collected?

Every specimen submitted for rabies diagnosis should be accompanied by a submission form containing the following data:

1. Specimen description

    a. Species

    b. Date of death and date of collection

    c. Date of shipment

    d. Location: name, geographical coordinates

2. Name and address of the owner or of the person who found the animal

3. Possible human exposure

    a. Date of exposure

    b. Type of exposure

    c. Location of the wound(s)

    d. Name and addresses of exposed people

4. Other information

    a. Age, sex and vaccination status of the suspect animal

    b. Clinical signs

    c. Animal contacts

2. Shipment of specimens

Whole carcasses, heads, brains or brain biopsies can be transported to the laboratory.

Ideally, fresh material should be transported to the specialized laboratory rapidly and under cold conditions.

If refrigerated/frozen shipment of samples is not possible, other preservation techniques may be used.
The choice of the preservative is dependent on the tests to be used for diagnosis:
i) Formalin-fixed specimens
Formalin fixation (10% [w/v] solution in phosphate buffered saline [PBS]) allows testing with FAT, immunohistochemistry, conventional and real-time PCR; however, modifications may be required and tests can be less sensitive compared to using fresh specimens. Formalin fixation inactivates the virus thus preventing virus isolation.
ii) Glycerol/phosphate buffered saline (PBS)
For transportation of specimens, infectivity may be extended for several days if diagnostic specimens are kept in a mixture of 50% glycerol in PBS. Glycerol/PBS slows bacterial action and therefore protects against the chemical and biological effects of putrefaction. Since rabies virus is thermolabile, this method does not prevent a decline in the viral load of the specimen. Under routine transport conditions in regions with high temperatures (above 30°C), this protection may only be effective for a matter of several days. Therefore, whenever possible specimens in glycerol/PBS should be kept refrigerated. As the virus is not inactivated by glycerol/PBS, all laboratory tests can be used to test these specimens.
iii) Preservation for molecular techniques
For molecular techniques, lysis buffers for nucleic acid extraction and RNA preservation buffers impregnated onto filter paper can be used. These buffers preserve rabies virus RNA and allow transport of specimens at ambient temperature without specific biohazard precautions for detection of viral RNA and further genetic characterization of rabies virus strains.

3. Choice of tests to be used

Whenever possible, all specimens should be examined using at least two different techniques: for example, the fluorescent antibody test (FAT) or the direct rapid immunohistochemistry test (dRIT) to detect antigen, the mouse inoculation test (MIT) or the rabies tissue culture inoculation test (RTCIT) as a back-up. (Molecular testing is also good practice in a capable laboratory, providing the operators have considerable experience and the results are interpreted with caution.)

The following tests can be performed according to the available specimens:

Specimen	Test
Fresh or frozen nervous tissue specimen	FAT or dRIT on smears, biological tests in mouse or mouse neuroblastoma cell cultures, RT-nPCR and One-step Real time-qPCR
Glycerol-preserved specimen, after removing excess glycerol	FAT or dRIT on smears, biological tests in mouse or mouse neuroblastoma cell cultures, RT-nPCR and One-step Real time-qPCR
Formalin-fixed nervous tissue specimens embedded in paraffin	FAT or dRIT following enzyme digestion (1), RT-nPCR and One-step Real time-qPCR

(1) Not recommended as a routine procedure

4. Laboratory diagnosis of rabies

Identification of the agent:

For the reliable diagnosis of rabies, the brain is required. Of the parts of the brain, the brainstem is the most reliable for the detection of antigen. Other tissues can also be used for rabies diagnosis, although not all are as reliable as brainstem. These include spinal cord, peripheral nerve tracts and salivary glands. The ante-mortem diagnosis of human rabies requires skin biopsies from the head region and corneal impression smears.

This section describes the gold standard test of rabies diagnosis: the fluorescent antibody test (FAT).

The direct rapid immunohistochemistry test (dRIT) can be used as an alternative method of FAT in routine rabies diagnosis as the tests have similar sensitivity and specificity, and this test can be used in laboratories without a fluorescent microscope.

Various molecular diagnostic tests, e.g. detection of viral RNA by reverse transcription PCR (RT-PCR) or real-time PCR, are used as rapid and sensitive additional techniques for rabies diagnosis. Although those molecular tests have the highest level of sensitivity, their use is currently not recommended for routine post-mortem diagnosis of rabies due to high levels of false positive or false negative results without standardisation and very stringent quality control.

- Collection of brain samples

- Fluorescent antibody test

- Molecular tests：

RT-nPCR

Real-time PCR

4.1 Identification of the agent

4.1.1 Collection of brain samples: drinking-straw method

Ideally, the brain is collected following the opening of the skull in a necropsy room, and the appropriate samples are collected, preferably brain stem, Ammon's horn, thalamus, cerebral cortex, cerebellum and medulla oblongata.

In field conditions, in cases where animals cannot be brought to the laboratory or when there is no access to laboratory facilities, it is possible to collect brain samples without opening the skull.

Precautions should be taken when handling central nervous system tissues from suspected rabies cases. Protective personal equipment (such as gloves, mask) should always be worn and precautions must be taken to prevent aerosols. Cutting tools, scissors and scalpels, should be used with care to prevent injury and contamination.

a. MATERIALS

Equipment/consumables

Protective clothing: gown, gloves and mask

Drinking straws or disposable plastic pipettes

Scissors and scalpel

Collection tube

b. PROCEDURE/METHOD

Drinking-straw method for brain sampling:

1. Bend the head of the animal so that the nose is touching the chest between the forelegs

2. This gives access to the occipital foramen

3. The straw is then introduced and inserted towards an eye
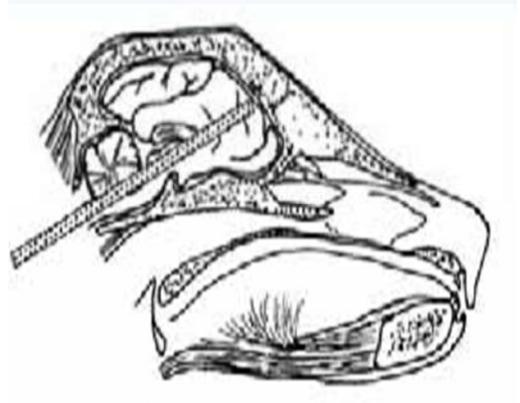
Area sampled by the straw

A 5 mm drinking straw or a 2 ml disposable plastic pipette is introduced into the occipital foramen in the direction of an eye. Samples can be collected from the rachidian bulb, the base of the cerebellum, hippocampus, cortex, and medulla oblongata. When using a straw it should be pinched between the fingers to prevent material escaping when withdrawing.

The different portions sampled with the straw are illustrated in this diagram:
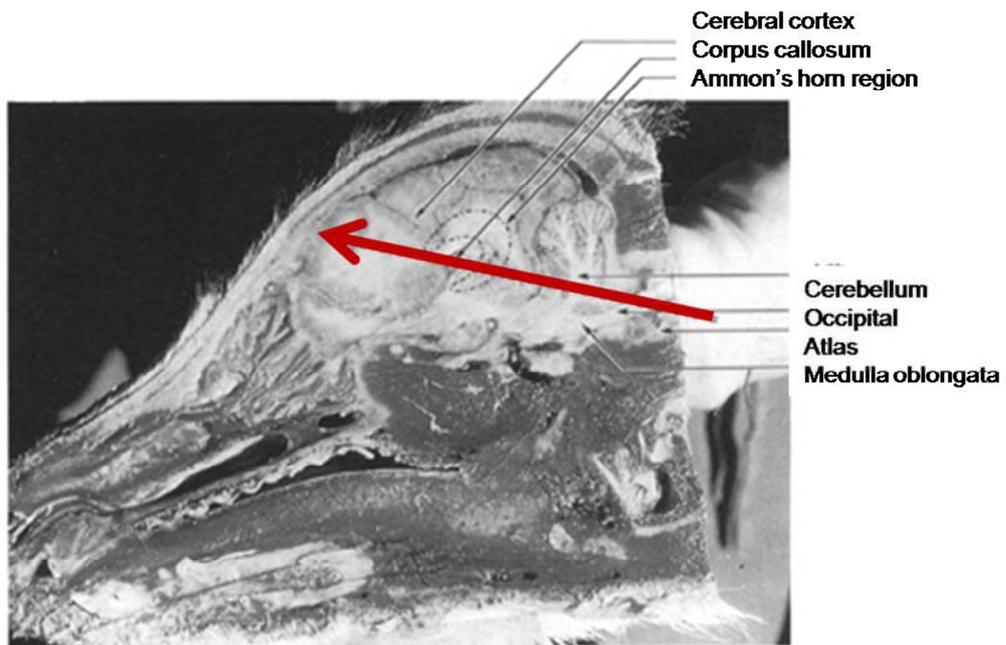
4.1.2 Fluorescent antibody test (FAT)

The direct fluorescent antibody test is recommended by both WHO and OIE as **the golden standard in experimental diagnosis**.

It is a rapid, sensitive and specific test that is not very expensive. It involves the staining of a tissue smear (usually brain) with an FITC-labelled anti-rabies immunoglobulin, following acetone fixation. The stained smear is washed in buffer and read under blue-light fluorescence microscope to detect the characteristic green fluorescence associated with rabies antigen corpuscles.

a. MATERIALS

Chemicals and reagents

FITC Conjugate (Bio-rad)

High-grade acetone, stored at -20°C

Distilled water

0.1M PBS pH 7.4

Tween-20

Equipment/consumables

Bio-safety cabinet

Freezer: -20°C and -80°C

Humidified incubator: 37°C (+/- 2%)

Fluorescent microscope

Waste container

Coplin jars

Wooden applicator sticks (tongue depressors, for lifting and retracting the brain)

Microscope slides

Micropipette tips

Slide boxes

Humidified box

Permanent marker pen

b. PROCEDURE/METHOD

1. Label the microscope slides. Label one slide with the date and the sample number.

2. Group small pieces of the selected brain sections on a wooden applicator stick and make an impression smear directly onto the slide.

The routine use of positive and negative controls is essential.

3. Air dry the smear thoroughly.

4. Place all slides into a Coplin jar containing cold acetone within the freezer for 30 min.

5. Remove the slides from the Coplin jar and rinse in PBST (1000mL PBS+0.5mL Tween-20) for 3 times.

6. Add 40µL of (Mixture of Bio-Rad Conjugate and 1% Evan's Blue) per slide.

7. Incubate at 35-37°C for 30-45 min in a humidified chamber (i.e. incubator).

8. Wash 2 times with PBST.

9. Air dry and apply cover slip, using mounting medium for fluorescence.

Smear to be stained

positive/negative control
Date

Sample number
Date
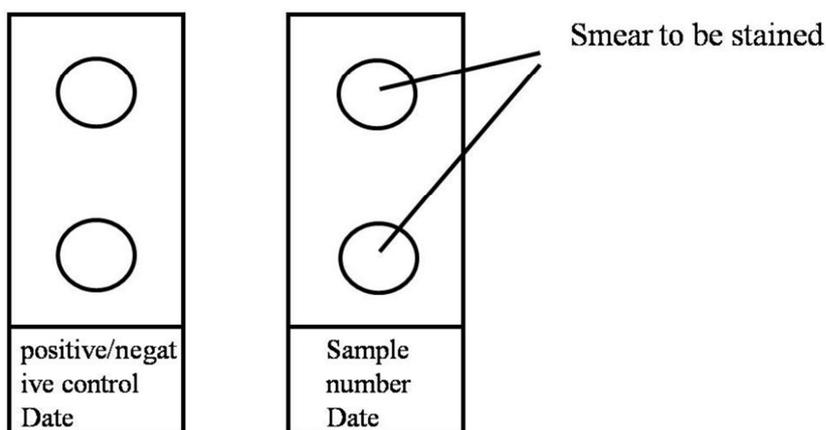
c. Reading
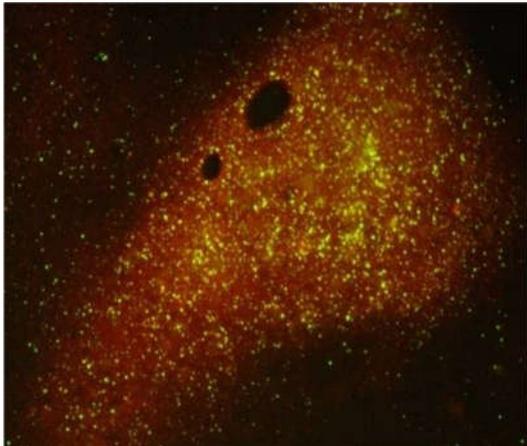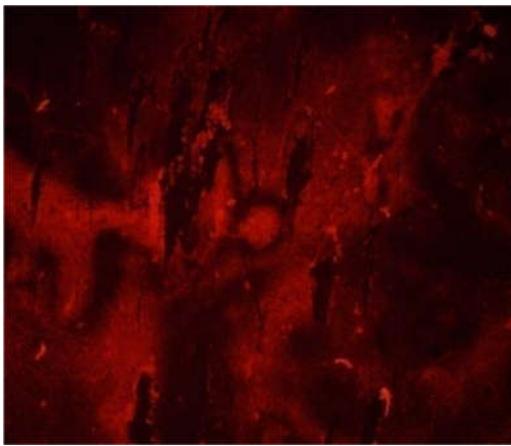
| Positive brain tissue | Negative brain tissue |

The slides should then be examined for specific fluorescence using an inverted fluorescence microscope filtered for the appropriate wavelength (490 nm and re-emits at 510 nm).

Slides are examined using a x10 eye piece and a x10 objective.

Generally, every slide should be examined independently by two experienced technicians who compare their results at the end of the reading session.

d. Result

Controls are read first, both positive (above left image) and negative (above right image) controls must be validated for tests.

Infected tissue appears as bright 'apple' green fluorescent inclusions. Dull green or red/green auto-fluorescent granules should not be counted as positive antigen.

4.1.3 Molecular test

4.1.3.1. RNA Extraction

Procedure is used for the extraction of viral RNA from both infected animal brain tissue samples and infected tissue culture. The extracted RNA is used in the nested RT-PCR and one-step real time PCR to detect rabies virus.

a. Materials

Chemicals and reagents

0.1M PBS pH7.4

RNA extraction kit

Equipment/consumables

Bio-safety cabinet

Pipettes (range from 10μL up to 1000μL)

Centrifuge (4°C centrifuge is recommended)

1.5 ml centrifuge tube (RNase and DNase free)

b. Procedure

i.   Sample preparation

Portions of brain collected from the hippocampus, thalamus, medulla oblongata and cortex are crushed with a grinder and grinding rod to obtain a 10%(w/v) suspension in PBS. Centrifuge at 10,000 rpm/min for 5 min. Transfer the supernatant into a new 1.5 ml tube.

Cell culture suspension is also available for RNA extraction if it is requested.

The same protocol should be used to prepare negative control with mouse brains and positive controls with pieces of positive brains of animals naturally-infected with RABVs.

ii.   RNA extraction

Total RNA can be isolated by using guanidinium isothiocyanate-phenol-chloroform based extraction methods (e.g. TRIzol®) or the

commercially available kits. The sensitivity of nucleic acid extraction reagents should be assessed before the molecular assay. Record all test details onto a worksheet to ensure traceability.

4.1.3.2 Nested RT-PCR (CVRI Document No: GB/T 36789-2018)

The reverse transcriptase (RT) polymerase chain reaction (PCR) is used to amplify a certain fragment of the virus genome (viral RNA). This protocol outlines the steps involved in the synthesis of *lyssaviruses* complementary DNA (cDNA) from viral RNA using the reverse transcriptase and amplification of viral cDNA for both diagnostic and research purposes.

a. Materials

Primer pairs

N127 (First round PCR) 5'-ATG TAA CNC CTC TAC AAT GG-3'

N829 (First round PCR) 5'-GCC CTG GTT CGA ACA TTC T-3'

RVN371F (Second round PCR) 5'-ACA ATG GAK KCT GAC AAR ATT G-3'

RVN371R (Second round PCR) 5'-CCT GYY WGA GCC CAG TTV CCY TC-3'

Chemicals and reagents

RNase free H_2O, dNTP Mixture (2.5mM), Random Primer (6nt) (50pmol/µL), Oligo(dT)$_{15}$ (50pmol/µL), M-MLV reverse transcriptase (200 U/µL), 10×M-MuLV buffer, RNase inhibitor (40 U/µL) (TAKARA), PCR Master mix (TIANGEN Biotech Co. Ltd.).

All PCR reagents are stored at –20°C.

Equipment/consumables

Pipettes (10-1000µL), vortex mixer, microcentrifuge, PCR system, microwave oven, gel mould, conical flask (250ml), gel electrophoresis tank/power pack, Gel Doc-IT imaging system, measuring cylinder

b. Procedure

i. Reverse Transcription of Rabies Viral RNA

1. Remove RT reagents from the freezer, keep them on ice, thaw and vortex them before use.

2. Prepare 12 µL RT reaction mix in a 0.2 mL PCR tube according to the reagents in table. Allow for pipetting variation by preparing a volume of master mix at least one reaction greater size than required.

Reagent	Volume Per Reaction (µL)
dNTP Mixture (2.5 mM)	4
Random Primer (50 µM)	1.5
Oligo(dT) $_{15}$ (50 µM)	0.5
M-MLV buffer (5×)	4
M-MLV reverse transcriptase (200 IU/µL)	1
RNasin (40 IU/µL)	1
Total volume	12

3. Add 8 µL sample, positive control RNA or negative control to the RT reaction mix within a PCR workstation in template room. The RT positive control is RNA extracted from the cell culture infected with fixed RABV strain CVS-11 (Challenge Virus Standard-11) and stored at -80 ℃. The negative control contains RNase-free ddH$_2$O.

4. Mix the contents of the RT tubes by vortexing, then centrifuge briefly.

5. Load the reaction tubes into a thermal cycler. Set up the cDNA synthesis program with the following conditions: 42 ℃ for 90 min, 95 ℃ for 5 min and 4 ℃ on hold. Set reaction volume to 20 µL. Start the RT run.

ii. First round PCR

1. Keep PCR reagents in table on ice in a clean room until use, then thaw and vortex them.

2. Prepare the first round PCR mix in a 0.2 mL PCR tube.

Reagent	Volume Per Reaction (µL)
PCR master mix	12.5
N127(20pmol/µL)	1
N829(20pmol/µL)	1
dd H$_2$O	9.5
Total	24

3. Add 1 µL sample cDNA into the first round PCR mix within a PCR workstation in template room. The PCR positive control is CVS-11 cDNA prepared in the above RT method. The PCR negative control is ddH$_2$O.

4. Transfer the sealed tubes to a PCR thermal cycler and cycle using the parameters as follows.

94°C for 2 min	1 Cycle
94°C for 30sec	
56°C for 30sec	35 Cycles
72°C for 40sec	
72°C for 10 min	1 Cycle
Hold at 4°C	

iii. Second round PCR

1. Prepare the second round PCR mix in a 0.2 mL PCR tube using the reagents in table.

Reagent	Volume Per Reaction (µL)
PCR master mix	12.5
RVN371F(20 pmol/µL)	1
RVN371R(20 pmol/µL)	1
dd H_2O	9.5
Total	24

2. Add 1 µL first round PCR product into the second round PCR mix. In addition, include ddH$_2$O as a negative control of the second round PCR.

   Perform the PCR thermal cycler using the same parameters as the first round PCR.

iv. Gel Electrophoresis

1. Prepare a 1.5 % agarose gel by adding 1.5 g agarose into 100 mL Tris-acetate-EDTA (TAE) and dissolving thoroughly by heating in a microwave oven.

2. Add ethidium bromide (EB) (final concentration 0.01 %) or other commercial EB substitution. Pour gel into the mould and leave the gel to solidify at ambient temperature for at least 30 min.

3. Prepare the loading samples by mixing 5 µL of each PCR product with 1 µL 6 × loading buffer.

4. Load the samples and suitable DNA marker separately into the wells, and run the gel for approximately 30 - 45 min at 120 V until the dye line is approximately 75 - 80 % down the gel.

5. Turn off power, disconnect the electrodes from the power source, and then carefully remove the gel from the gel box.

6. Use a UV gel documenting device to visualize and photograph the DNA fragments.
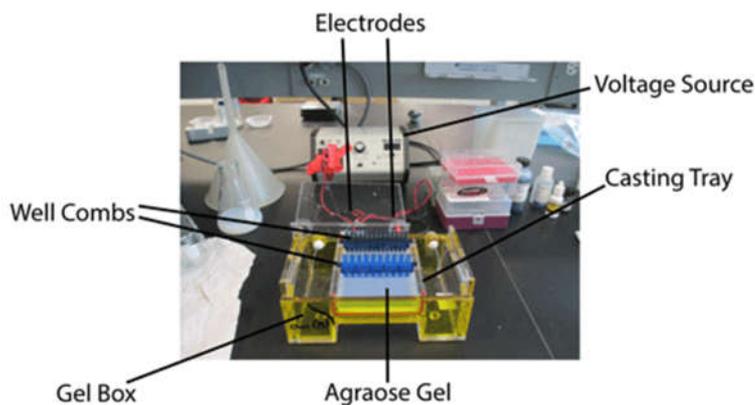
c. Results

A positive PCR result is observed in the form of a bright band; the size of the PCR product obtained can be confirmed by comparing its position against the bands of the marker, which are of known size (refer to data sheet provided with the marker for size of bands). The positive controls should also produce bands, whereas the negative controls should produce no band.

Let take the image blow for example, the marker provides the size of bands from 100bp to 2000bp. The first round PCR yields 845bp amplicons and the second round PCR produces 374bp amplicons.

It is a good practice to check if positive and negative controls are correct before making a judgment on the sample(s).

In the example above, for the first round PCR, the negative control (-ve) should produce no band while the positive control (+ve) obtains a bright band (between 750bp to 1000bp). The PCR result shows that sample 1 is negative and sample 2 is positive.

For the second round PCR, the negative control (-ve) should produce no band; the positive control (+ve) obtains a bright band (between 250bp to 500bp). So sample 5 is negative and sample 6 is positive.

Typically, if the first round PCR shows a positive, the second round PCR is usually positive. Then we confirm the sample as positive.

If the first round PCR doesn't show a positive but the second round PCR is positive, we can also confirm the sample is positive.

4.1.3.3 One-step Real-time qPCR (VRI Document No: VRI/TM/MV029)

Rabies virus and rabies-related viruses are members of the *Rhabdoviridae* family, genus *Lyssavirus*. The nucleic acid sequence of N protein (part of which is the target for this assay) displays a high degree of homology between species and is therefore, an excellent target for detection of the virus in field samples.

The reverse transcription (RT)-polymerase chain reaction (PCR) is used to amplify the N gene of the virus genome (viral RNA). Real-time PCR has been developed to increase sensitivity and to obtain results faster.

With this procedure, the RNA of rabies virus can be enzymatically amplified as cDNA copies using reverse transcriptase (RT). The cDNA copy of rabies then can be amplified using polymerase chain reaction (PCR) and the amount of DNA formed after each cycle detected with fluorescently-tagged oligonucleotide probes. This technique can confirm FAT results and can detect rabies virus in tissue samples, such as brain tissue, saliva, CSF (Cerebrospinal Fluid) and suspension of cell culture.

Standardisation and very stringent quality control are required. As PCR can produce false positive or false negative results it should only be used in combination with other conventional techniques.

The protocol has been validated to detect rabies viral RNA for genotype I.

a. Materials

Primer pairs and fluorescently-tagged oligonucleotide probes

RABVD1 For: 5'-ATGTAACACCYCTACAATG-3'

RABVD1 Rev: 5'-GCMGGRTAYTTRTAYTCATA-3'

RABVD1 Probe: FAM-5'-CCGAYAAGATTGTATTYAARGTCAAKAATCAGGT-3'-BHQ

Equipment/consumables

Pipettes (10-1000μL)

Vortex mixer

Microcentrifuge

Real-time PCR system

b. Procedure

i. Preparation of Real-time PCR master mix

1. Calculate the total number of reactions (including positive and negative controls).

2. Prepare a master mix containing the following reagents. (**Note**: all enzymes are handled on ice)

Reagent	Volume Per Reaction (µL)
2X PCR Buffer	12.5
25 X RT PCR Enzyme Mix	1.0
Nuclease-free water	2.75
RABVD1 For (18 µM)	1.25
RABVD1 Rev (18 µM)	1.25
RABVD1 Probe (5µM)	1.25
Total volume	20

3. Cap the tube and invert or vortex it several times to mix the reaction components.

4. Centrifuge the tube briefly to remove the bubble.

ii. Load the tubes

1. Transfer 20 µL of PCR reaction mix into PCR tubes. Add 5 µL RNA for each tube.

2. Seal the plate with the appropriate lids.

3. Label (avoid label on the lid) the tubes clearly with date and sample identification. One of the samples will be positive mouse brain RNA (positive control) which was extracted at the same time as the test samples.

4. Centrifuge the plate briefly.

5. Load the plate into the instrument.

iii. Incubate the reactions using the following thermocycling profile

45°C for 10 min	1 cycle

95°C for 10 min	1 cycle
95°C for 15 sec	45 cycles
50°C for 45 sec	
Hold at 4°C	

c. Results

For each real-time PCR, a critical threshold cycle number (Ct) is determined corresponding to the PCR cycle number at which the fluorescence of the reaction exceeds a value determined to be statistically higher than background by software associated with the PCR system.

Positive control: 30<Ct<34,

Negative control: no Ct,

then the PCR works well and there is no contamination in the process of preparation of PCR master mix and template addition.

For reading the samples：　Ct≤40 shows that the sample is positive;

Ct＞45 shows that the sample is negative;

40＜Ct＜45 shows that the sample is indeterminate.

4.1.4 Phylogenetic analyses

4.1.4.1 Amplification of N and G genes

The N and G genes of rabies virus (RABV) are 1,353 bp and 1,575 bp in length (Figure 1), and encode structural nucleoprotein (N protein) and glycoprotein (G protein). N protein is an essential component of nucleocapsid that wraps RNA genome, and G protein forms individual spikes on the surface of viral envelope and function in virus attachment and penetration. Usually, N gene is used to phylogenetic analysis to inspect relationship of virus to other members. This protocol shows how to determine the complete genes of N and G from cDNA that is positive for RABV and the steps to phylogenetic inspection of RABV using N gene.

Figure 1. A gene map of RABV is used to show the sizes of N and G genes as well as their amplicons' sizes.

a. Materials

Primer pairs

NF(forward): ACGCTTAACAACAAAAYCADAGAAG

NR(reverse): GGRTTGACGAARATCTTGCTCAT

GF(forward): CGCTGCATTTYATCAAAGTCAAG

GR(reverse): TGMAYGGAGTTCAAGGAGGAC

Chemicals and reagents

ddH$_2$O, Phusion DNA polymerase (NEB), dNTP Mixture (10 mM).

All PCR reagents are stored at –20°C.

Equipment/consumable

Pipettes (10-1000µL), Vortex mixer, microcentrifuge, PCR system, microwave oven, gel mould, conical flask (250ml), gel electrophoresis tank / Power pack, gel Doc-IT system, measuring cylinder

b. Procedure

1. Prepare reaction mix in a 0.2 ml MicroAmp tubes as follow, keeping all reagents on ice.

Reagent	Volume Per Reaction (µL)
Phusion DNA polymerase	0.5
5×HF Buffer	10
Forward primer（10pmol/µL）	2.5
Reverse primer（10pmol/µL）	2.5
dNTP Mixture (10mM)	1
dd H$_2$O	30
DMSO (supplied)	1.5
cDNA	2
Total	50

2. Mix gently with pipette and close the tubes' lid tightly, then label the lid clearly. Transfer tubes to PCR machine.

3. PCR programs:

N gene:

98°C for 30sec	1 Cycle
98°C for 10sec	35 Cycles
52°C for 30sec	
72°C for 45sec	
72°C for 10 min	1 Cycle
Hold at 4°C	

G gene:

98°C for 30sec	1 Cycle
98°C for 10sec	40 Cycles
56.7°C for 30sec	
72°C for 50sec	
72°C for 10 min	1 Cycle
Hold at 4°C	

Gel electrophoresis

See Rabies Virus RT-nested PCR Detection.

c. Results

The expected results of electrophoresis are shown in figure 2. The amplicons of N and G genes should be 1,536 bp and 1,778 bp, respectively. Their positions can be roughly read against the Marker. But negative samples cannot obtain such amplicons. These amplicons can be used directly to Sanger sequencing.
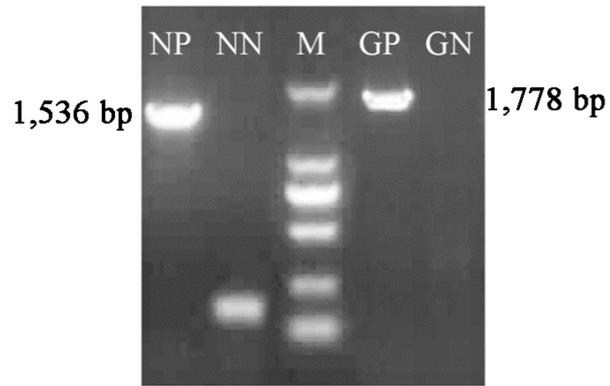
Figure 2. Electrophoresis analyses of PCR amplification of N and G genes. NP, positive amplification of N gene; NN, negative amplication of N gene; M, DNA marker DL2000, the bands from top to bottom are 2,000 bp, 1,000 bp, 750 bp, 500 bp, 250 bp, and 100 bp; GP, positive amplification of G gene; GN, negative amplication of G gene.

4.1.4.2 Phylogenetic analysis of N gene

N gene of a Chinese isolate (Buffalo/China/Hubei070308) was used as the query sequence, to find out its phylogenetic relationship with other Chinese isolates.

a. Materials

Data set*

S.N.	Country	GenBank accession number	Isolate
1	China	NA	Buffalo/China/Hubei070308
2	Bangladesh	AB699219.1	BDR7
3	Bangladesh	AB699214.1	BDR1
4	Bangladesh	AB699220.1	BDR5
5	Bhutan	AB910532.1	Btn115
6	Cambodia	EU086170.1	9914CBG
7	Cambodia	KM366224.1	K0208220
8	Cambodia	KM366249.1	P0407547
9	Cambodia	KM366255.1	V0926624
10	China	EU159380.1	WH5
11	China	DQ666319.1	Hunan_Xx35
12	China	DQ515993.1	Hunan_HuNDN16
13	China	EU828655.1	GC07
14	China	EU828651.1	WJ07_2
15	China	EU828653.1	GN07
16	China	EU159386.1	GX4
17	China	KT221095.1	Chongqing_CQWSD01
18	China	KT221097.1	Chongqing_CQQJD6
19	China	KT221100.1	Hunan_HuND02

20	China	JF819603.1	Yunnan_CYN0818D
21	China	JF819610.1	Yunnan_CYN0812D
22	China	DQ496219.1	Hunan_HuNPN01
23	India	KX434489.1	IAP_R91
24	India	KX434517.1	IUP_R197
25	India	KX148246.1	97002IND
26	India	KX434506.1	ITN_R148
27	Indonesia	KX148266.1	03003INDO
28	Indonesia	JX462623.1	BL659_09
29	Indonesia	JX462620.1	BL598B_09
30	Laos	EU086193.1	9910LAO
31	Laos	AB981671.1	Lao11
32	Laos	AB981666.1	Lao3
33	Mongolia	AB571004.1	MGL_22
34	Mongolia	AB570995.1	MGL_2
35	Mongolia	AB571002.1	MGL_20
36	Mongolia	AB571006.1	MGL_24
37	Myanmar	EU086166.1	9915BIR
38	Myanmar	EU086164.1	9909BIR
39	Myanmar	EU086165.1	9913BIR
40	Nepal	EU086196.1	9901NEP
41	Nepal	JX944565.1	4403_13(09030NEP)
42	Nepal	KM979364.1	NPDOG12_1
43	Pakistan	HE802676.1	Pk_24
44	Pakistan	HE802675.1	Pk_23
45	Philippines	EU086202.1	94280PHI

46	Philippines	EU086203.1	03006PHI
47	Philippines	EU086204.1	03007PHI
48	South Korea	NA	SKRBV9801YC
49	South Korea	AY730597	KRC5
50	Sri Lanka	AB041969.2	SRL1145
51	Sri Lanka	AB569299.1	H_08_1320
52	Sri Lanka	LC110291.1	H_547_12
53	Thailand	AB178894.1	THA1017
54	Thailand	EU086208.1	8738THA
55	Vietnam	EU086210.1	01017VNM
56	Vietnam	EU086209.1	01016VNM

*There is no reference sequence of Korea DPR and Malaysia available in any public database.

Bio-software

Web BLAST, MEGA7.0

b. Procedure

1. Use N gene of Buffalo/China/Hubei070308 as query sequence to preliminarily find its close neighbors using **Web BLAST** (Figure 3);

Figure 3. Interface of **web BLAST**, there are many options to BLAST, **Nucleotide BLAST** is designed to search the most similar nucleotide sequences in public database using nucleotide sequence as query sequence; **BLASTx** can translate nucleotide into protein then search the most similar protein sequences, while **tBLASTx** does the opposite job, search the most similar translated nucleotide using protein; the last tool is **Protein BLAST** that can directly search protein using protein.

2. Click **Nucleotide BLAST** to activate **blastsuite** (Figure 4);
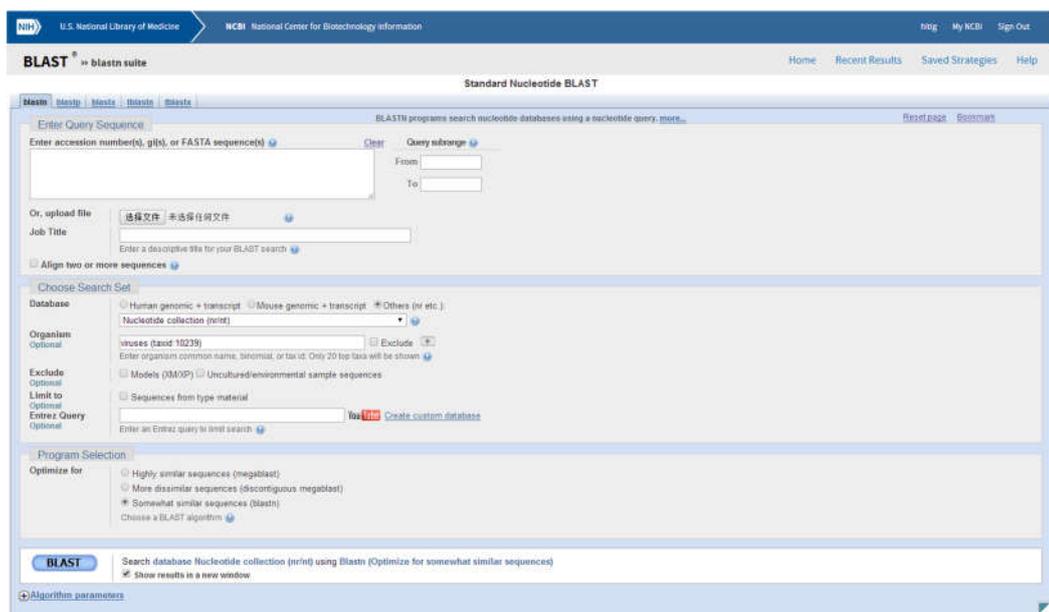
Figure 4.　The overview of blast suite.

3. Enter Query sequence into the box; please note that there are three recommended sequences formats, Genbank accession number(s), gi(s), or FASTA sequence(s). Besides query sequence can be directly paste into the box. Then specify options by choosing **Other** database in Search Set and **Highly similar sequence** in Program Selection. Finally, click BLAST button. Detailed operation is shown in Figure 5.
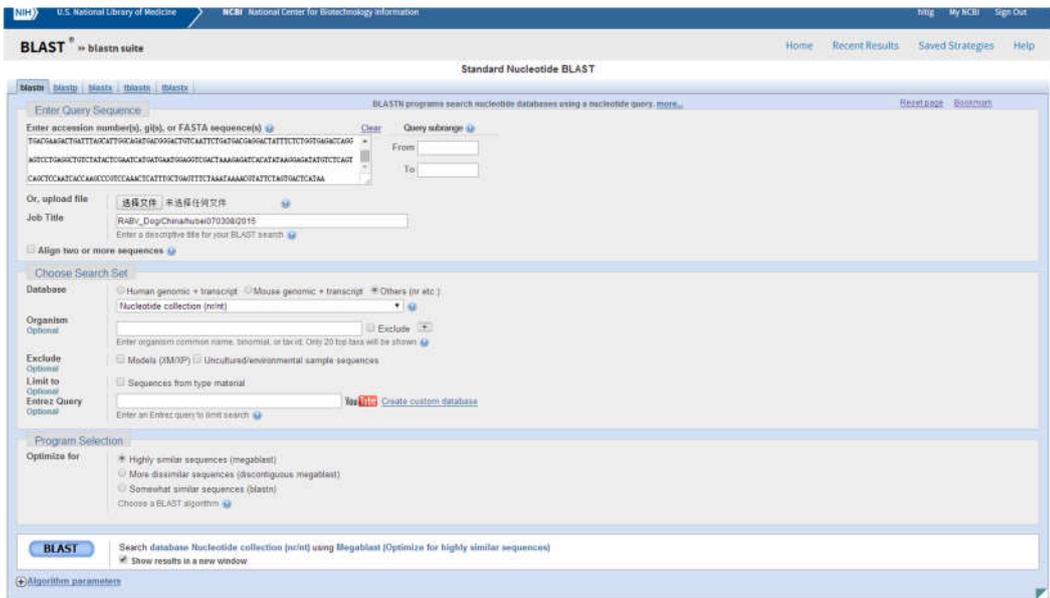
Figure 5. Enter query sequence into the box by directly pasting and specify BLAST search options.

4. BLAST will perform searching and result will be automatically updated in certain time until the analysis is done (Figure 6).

Figure 6.   BLAST is performing analysis.

5. From BLAST results we can clearly see which sequence is the most similar to query sequence. Here we found that the query sequence is 100% identical to Rabies virus isolate hubei070308 (this is the exact isolate we used as query sequence) then 99% to other Chinese isolates (Figure 7)
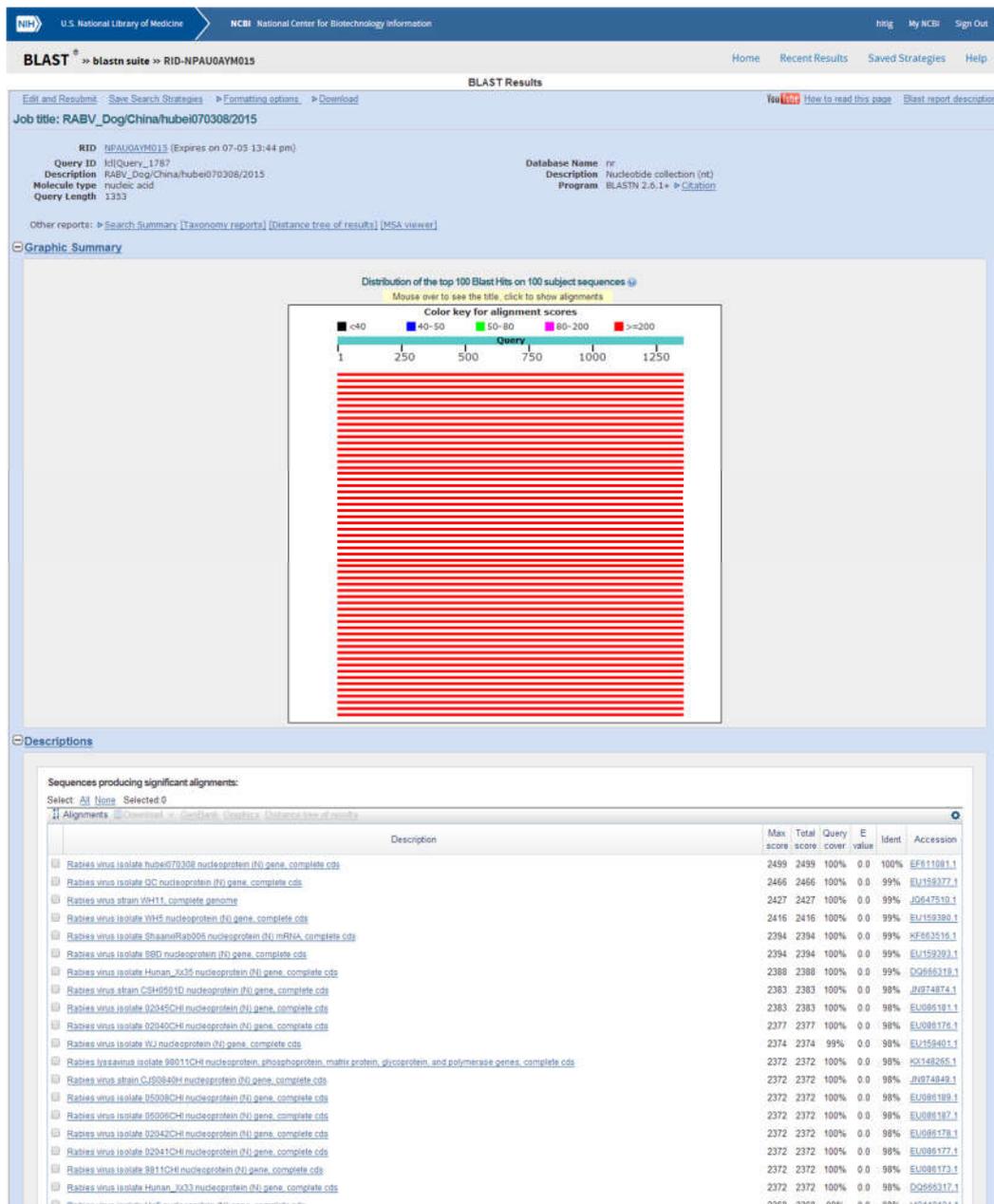
Figure 7.   The overview of BLAST result.

6. According to BLAST result, we can randomly choose and download several sequences that can be used as reference sequences in the follow-up intensive phylogenetic analysis. Here we download 5 sequences, WH5, HuNDN16, Xx35, GC07 and WJ07. Please note that the downloaded sequences must be FASTA format.

7. Before conducting intensivephylogenetic analysis, please make sure that *MEGA* 6.06 has installed in your computer. Please double-click the*MEGA* icon to activate the software (Figure 8).
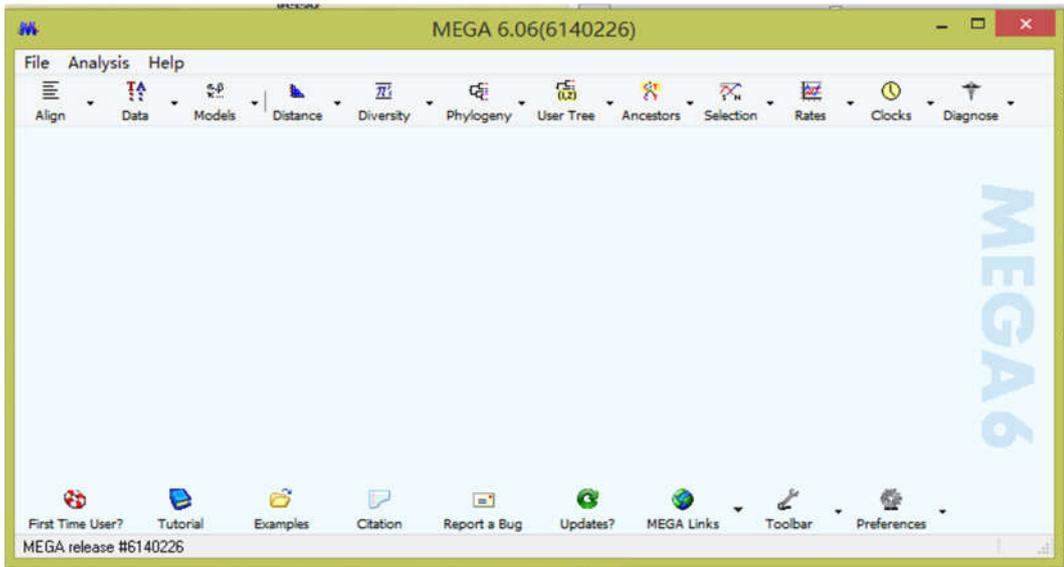
Figure 8.   The main interface of **MEGA**.

8.   Click **Align** to provide a drop-down menu with a list of selectable options, and choose **Edit/Build Alignment** (Figure 9).
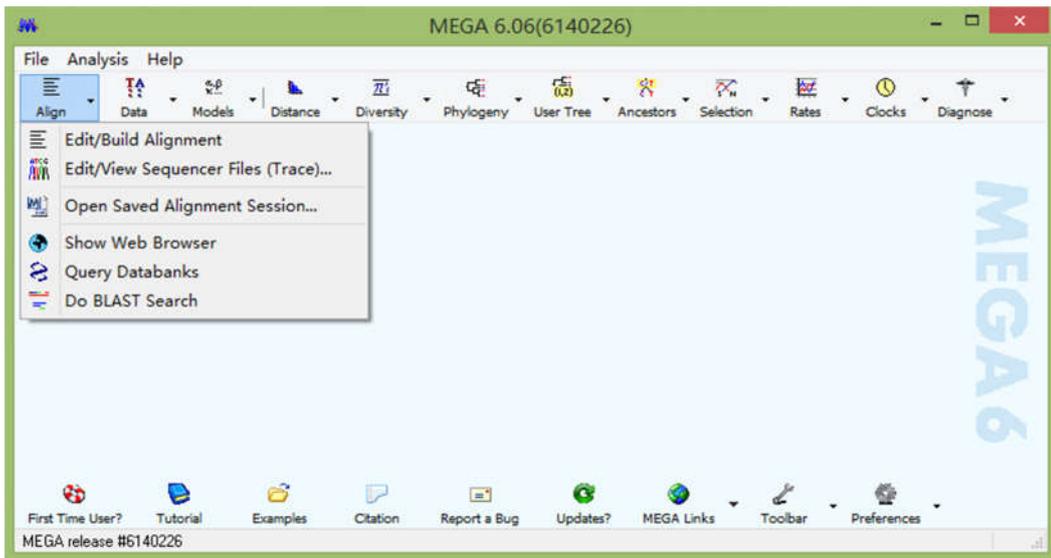
Figure 9.   The list of options in the drop-down menu of **Align**.

9.   Then you will be asked to select an option, here we choose **createa new alignment**, then we click DNA to specify datatype for alignment (Figure 10).
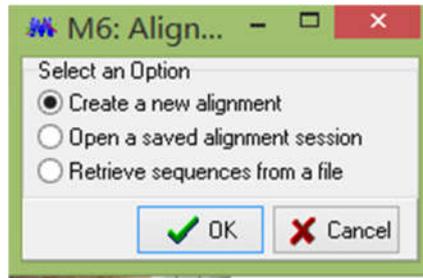
Figure 10. Options to analysis.

10. Then a new worktable (**Alignment Explorer**) will show up, we can directly drag FASTA file into worktable and click **W** in main menu to popup options, here we choose **Align DNA**,another sequential two popup menus will show up, click **OK** and use these default parameters (Figure 11).
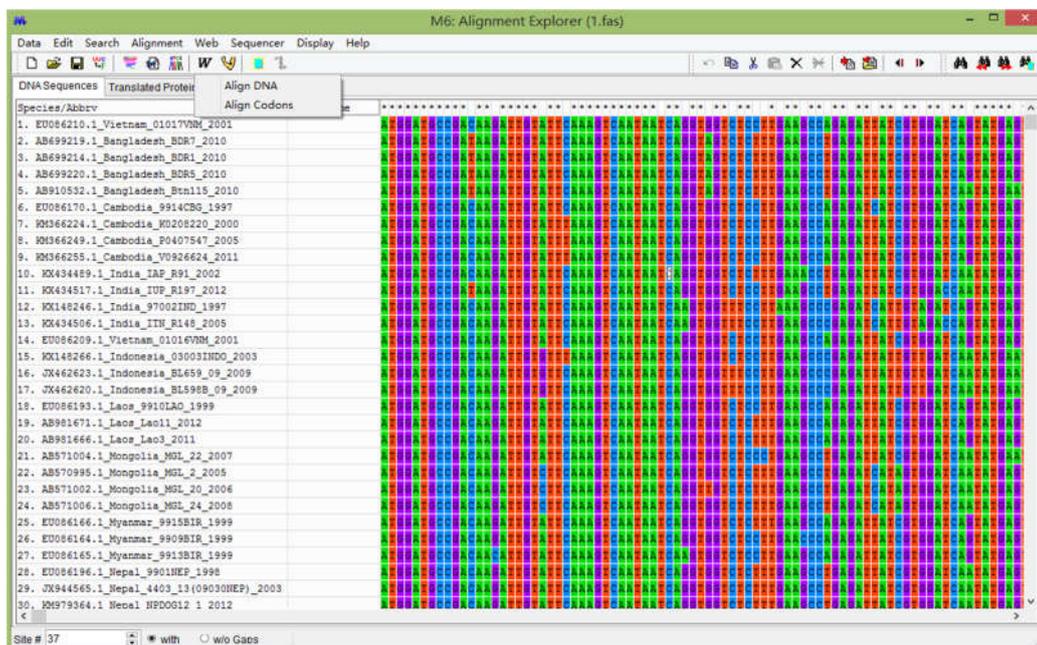
Figure 11. Sequences are input into Alignment Explorer, and **Align DNA** is chosen to align sequences.

11. MEGA will drive **ClustalW** to align these input sequences. It may take a few minutes depending on the size of data set (Figure 12).
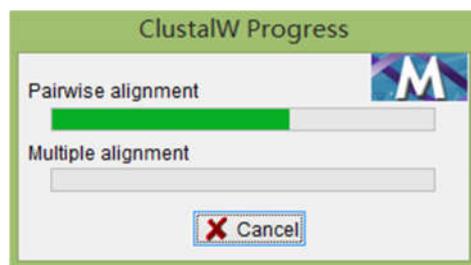
Figure 12.Alignment is in progress

12. When done, go to main menu of **AlignmentExplorer** and click **Data** to pull down a drop-down menu, then click **Phylogenetic analysis** and further confirm this operation by click **Yes** button in a new popup (Figure 13).
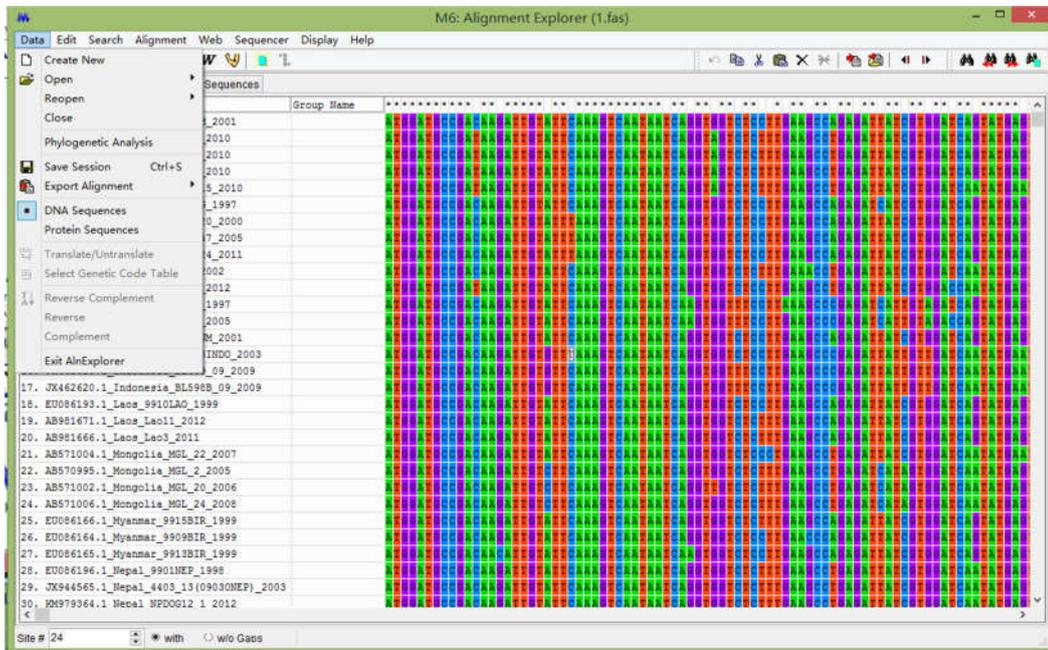
Figure 13. Choose **Phylogenetic Analysis** in the drop-down menu of **Data**.

Then go back to the main interface, click **Phylogeny** to choose **Construct/TestNeighbor-Joining Tree** (Figure 14).
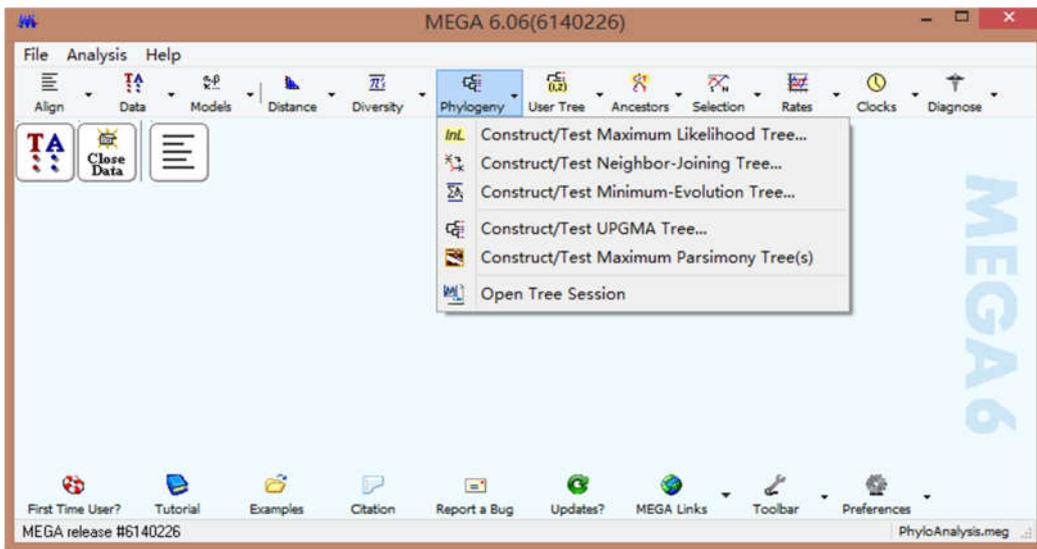
Figure 14. Specify method to draw phylogenetic tree.

13. Specify ***Bootstrap method*** in ***Test of Phylogeny***, 1000 in No. of Bootstrap Replications. Other options use default parameters, and then click Compute to start tree drawing (Figure 15).
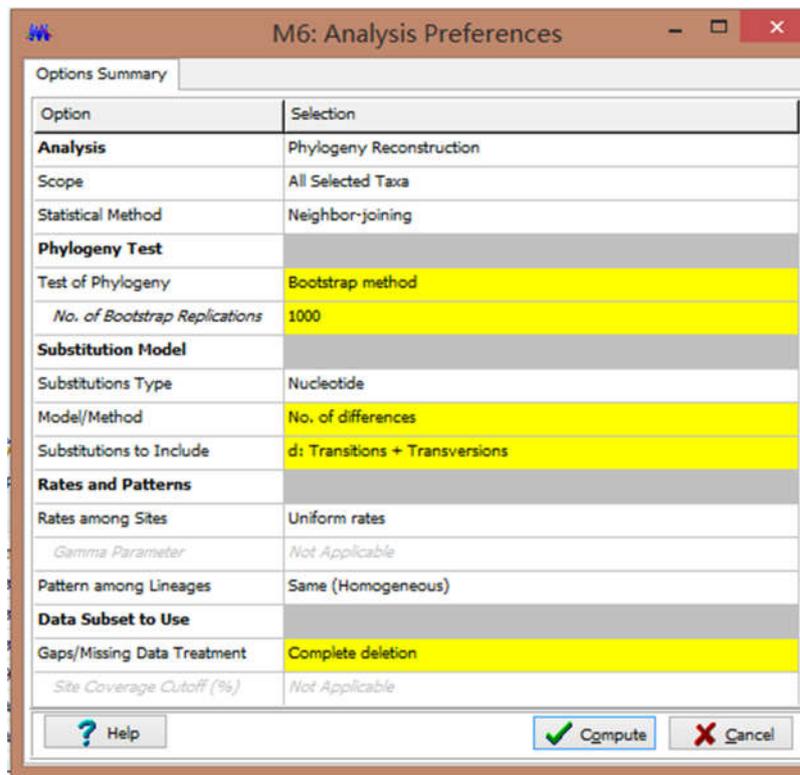
Figure 15. Choose 1000 of ***Bootstrap method*** to draw phylogenetic tree.

14. Finally, a neighbor-joining tree is completed (Figure 16).
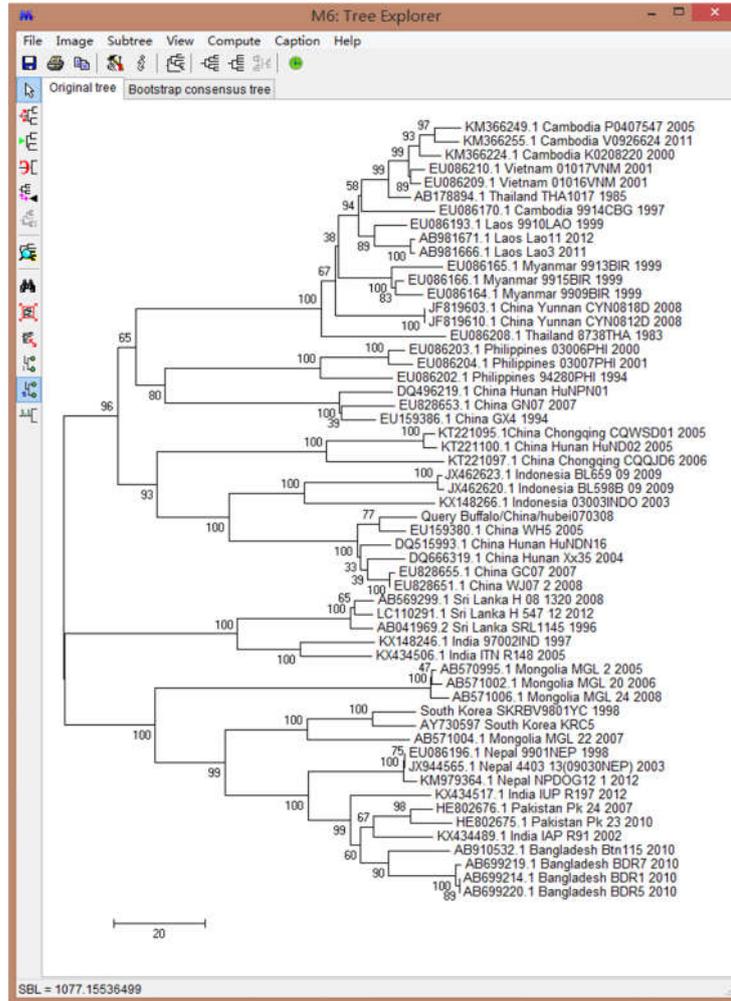
Figure 16.   A phylogenetic tree will present in Tree Explorer.

15. This tree can be usually accepted to publish in a majority of journals. Also, this tree can be edited to highlight some isolates, see Figure 17.
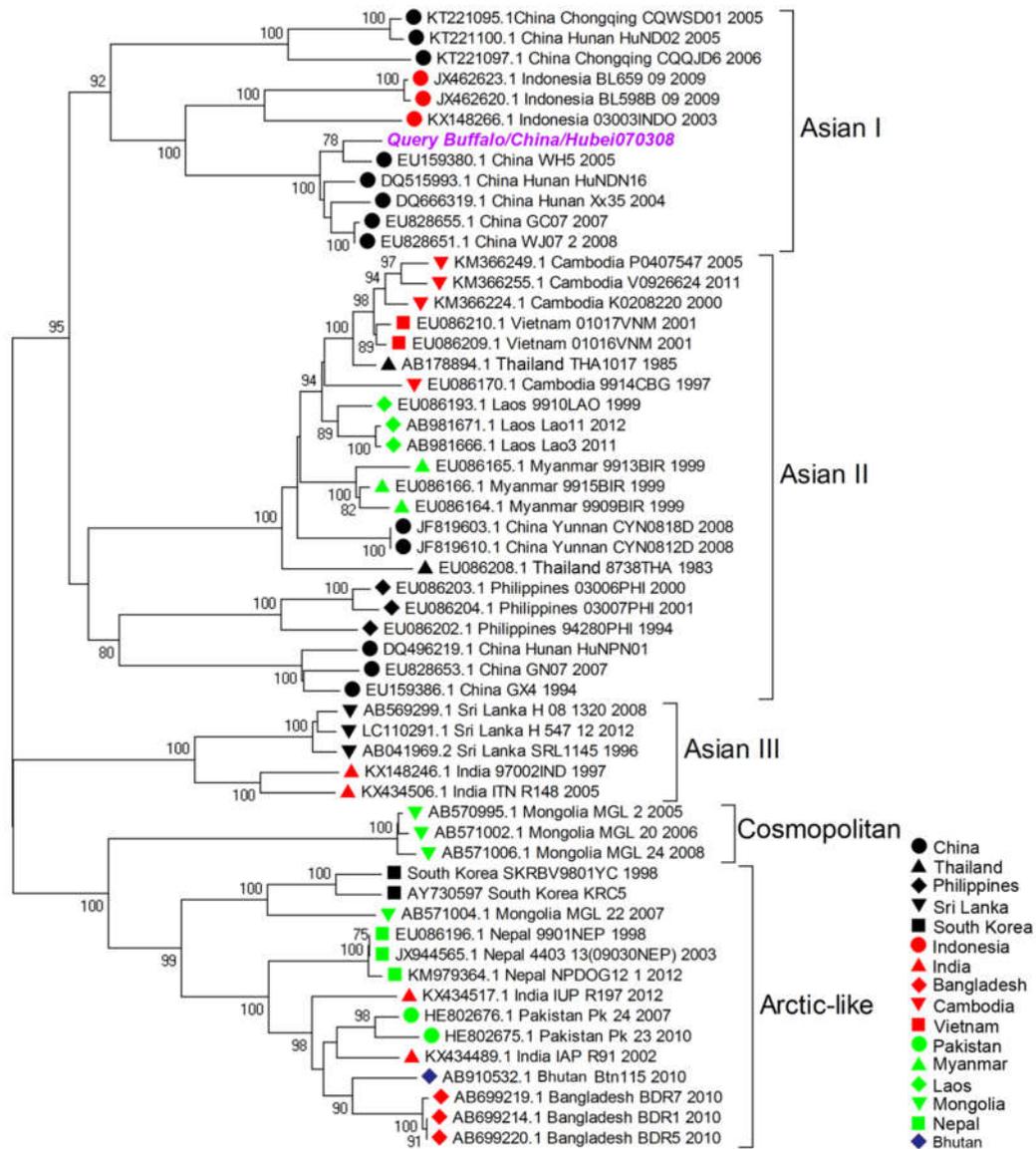
Figure 17. Phylogenetic analyses of N genes of representatives of Rabies virus from several Asian countries.

c. Results

A total of 56 isolates' N genes from 16 Asian countries were included in this phylogenetic analysis. Generally, these isolates can be classified into 5 phylogroups, Asian I, Asian II, Asian III, Cosmopolitan, and Arctic-like. All Chinese isolates involved here fall into Asian I and Asian II phylogroups, and Buffalo/China/Hubei070308 shows very close evolutionary relationship with isolates from Wuhan (WH5), Hunan (HuNDN16), phenotypes of Asian I. As to members of phylogroups, Indonesian and Chinese isolates composed Asian I group, while isolates from 7 Asian countries: Cambodia, Vietnam, Thailand, Laos, Myanmar,

China, Philippines, make up to Asian II group, and indicating wide distribution in this area. Asian III mainly consists of isolates from Sri Lanka and India, but some Indian isolates along with these of South Korea, Mongolia, Nepal, Pakistan, Bhutan, and Bangladesh comprise Arctic-like group. Of note is that several isolates of Mongolia is classified as Cosmopolitan group.

5. Management of biological safety in the laboratory

Lyssaviruses are Group 3 pathogens i.e. "organisms that may cause severe human disease and present a serious hazard to laboratory workers; they may present a risk of spread in the community but there is usually effective prophylaxis or treatment available".

Human deaths have resulted from infection with each of the *lyssavirus* species except Lagos bat virus, Irkut, Khujand, Aravan and the putative Shimoni; however even these viruses are known to cause an infection in other animals which is clinically indistinguishable from rabies. Therefore, it must be assumed to be capable of causing fatal encephalitis in humans.

The risk of acquiring a rabies virus infection in the laboratory is related to the techniques undertaken. Rabies diagnosis in field specimens may present a greater risk than the production of fixed strains of rabies on well-defined substrates, because field strains may have undetermined characteristics and also because such specimens may carry other unknown pathogens.

Every laboratory must develop its own working routine when dealing with pathogens, but there are some general principles that are summarised here. These risk limiting measures include:

5.1 Maintenance of integrity in "safe" and "high-risk" working areas

High-risk areas, which may comprise only the necropsy room or include different rooms where rabies diagnosis is performed, must be clearly separated from safe areas.

Access to the high-risk areas of the laboratory should be restricted only to those personnel who are vaccinated for rabies and who are familiar with the laboratory's safety protocols.

Staff should not enter a high-risk area without wearing protective clothing. This protective clothing must not be worn in a "safe" area. Clothing from a contaminated area must be decontaminated (e.g. by autoclaving) before removal for laundry. One method of ensuring that security is not breached is to wear colour-coded laboratory clothing, i.e. white clothing for safe areas and a coloured tag for the high-risk area.

Contaminated paperwork associated with a rabies specimen may represent a hazard to unvaccinated staff. If packages are to be opened in the post-mortem room, registration

documents should be completed and removed from the room before the commencement of necropsies.

Apparatus (tabletops, vice, socket forceps, scissors, forceps and grinder, etc.) used in the post-mortem room must be disinfected and cleaned between specimens. A sterile set of instruments should be used for each specimen and contaminated instruments should be disinfected by boiling, autoclaving or adapted chemical treatment. All contaminated surfaces and instruments should be washed and disinfected after every diagnostic session and the floor, walls and other structures of the post-mortem room should be disinfected and washed down daily (or routinely). Potentially infectious material such as brain smears should be fixed before removal from the post-mortem room, but it should be remembered that acetone fixation does not totally inactivate rabies virus.

5.2 Immunisation of staff

WHO recommends the preventive pre-exposure immunisation of staff that work with live rabies virus.

The immunisation protocol includes three injections at days 0, 7 and 28 and a booster injection one year later and then every 3 years in the absence of serological follow-up. Serological confirmation of protection should be carried out 10 days or more after the last injection. The subsequent testing of antibody level is recommended annually and staff whose antibody level declines below 1.0 IU/ml should be given a booster vaccination.

5.3 Physical protection from biohazardous agents

Good laboratory practice is the first line of defence against accidental infection.

Wearing of protective clothing, consisting of gowns, aprons, gloves, visors, masks, goggles and boots will protect the operator's skin and mucous membranes against splashing and spillage. Operation processed in the biosafety cabinet is recommended when dealing with tissue samples without opening the skull. All protective clothing is removed when leaving the rabies laboratory and stored near the exit.

Contaminated documents that contain information requiring transmission to other areas should be photocopied in a plastic envelope or decontaminated (for instance with ultra-violet light).

Storage and consumption of food or drink, smoking and applying cosmetics should be prohibited in the laboratories.

6. Disposal of biohazardous waste

Rabies virus is easily inactivated by heat or chemical treatment.

5% NaOH are some of the most commonly employed disinfectants.

Diagnostic operations produce waste that is infected or contaminated with rabies virus. Safe disposal of these wastes is an important aspect of laboratory management. Although incineration is the most effective means of destroying animal and biohazard waste, it requires a lot of energy. Waste can be autoclaved at 121℃ for 30 min before it is removed from the laboratory. An alternative is to bury this waste in quicklime in a pit inaccessible to the public and to scavengers, in compliance with local legislation.