

**Title: Construction of laboratory net work on the molecular epidemiology and the development and standardization of Rabies diagnostic methods**

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**Summary: 300~500 words**

Rabies is a fatal zoonotic disease with almost 100% fatality rate. As both reservoir hosts and transmitting vectors, variety of animals maintain and spread this disease worldwide, especially the dogs have been taken as important vector for rabies attacking human beings. During the recent years, the number of human rabies cases increased consecutively in China. Since 2003, there have been more than 2000 human rabies cases annually, of which most occurred in Guizhou, Guangxi and Hunan provinces. Despite carrying out several epidemiologic investigations about the factors resulting in high incidence rate, we lack research on pathogeny all the same. Therefore, it is necessary to take investigations on molecular properties about dogs infected with rabies virus in high-incidence regions, to discuss properties, origins and variations of rabies virus, and so on. This provides pathogenic evidences for effective control and prevention of rabies spread in China.

To investigate factors contributing to the current rabies epidemic in China, we collected 2887 dogs' brain specimens from 1-2 cities respectively selected out of regions with high-, middle- and low-incidence rate in Guizhou, Guangxi and Hunan provinces which suffered from rabies most heavily from Oct. 2005 to Sep. 2007. The brain tissue were detected with both direct immunofluorescence assay (IFA) and

RT-PCR. Finally we got 66 positive dog brain samples and the positive rate was 2.3%. In addition, two dog brain samples and one human canthus sample infected with rabies virus were detected likewise. By amplification and sequence, the partial N gene(634-1353 N-coding nucleotide) was obtained from 56 positive samples. Sequences comparison showed that they share a high degree of homology in nucleotide acid(87.6% -100%) and deduced amino acid(95.8% -99.6%). This indicates that the variations in this region are synonymous mutations.

The analysis of phylogenetic trees indicate that all of the 56 positive samples belong to genotype 1 Lyssavirus and distribute regionally. In despite of a few crossover among adjoined provinces, the investigated samples are basically divided into 3 subgroups ( I 、 II 、 III ) relying on provinces from which they were collected. However, branch made up of Hunan samples is earlier than that of Guangxi and Guizhou, combining with the increased number of dogs used as food and pets, the increased dog trade and the low immunity rate, we deduce that , in recent years , maybe, the viruses from Hunan province has spread to Guangxi and Guizhou, which coincides with the transmission of human rabies cases from Hunan to Guangxi to Guizhou in 2001-2005. Therefore, the changes of rabies epidemic regions may be caused by viruses' transmission. In subgroup III, there are only 7 samples(6 from Guizhou and 1 from Guangxi), which shows a close relationship with that from wide animals internationally. We deduce that there are rabies from wide animals in Guizhou, which is another reason that why this region had a sharp rise incidence in recent years.

The results indicate that the variation of N gene of rabies virus from high-incidence regions has geographic characteristics, and takes on a transmitting trend from Hunan to Guangxi and Guizhou. It is deduced that the consecutive high-incidence in these three provinces correlates with spread of rabies virus; the sharp rise of rabies in Guizhou may be caused by the variety of origins of rabies virus. In conclusion, reduction and elimination of animal rabies is where the shoe pinches for control of human rabies.

**Purpose:**

1. Elucidate etiological characteristics of the epidemic rabies continuing in China.
2. Establish the standard diagnostic systems for the strengthening of national rabies surveillance.

**Methods:**

Specimen collection: Randomly collected 2887 brain specimens from domestic dogs in restaurants of 15 cities in Guizhou, Guangxi, and Hunan provinces.

Rabies virus detection: All specimens were examined with a direct immunofluorescence assay (DFA) with fluorescence-labeled monoclonal antibody directed against the rabies virus N-protein (Rabies DFA Reagent; Chemicon Europe Ltd, Chandlers Ford, Southampton, UK), positive or suspect rabies specimens were further evaluated by RT-PCR.

Sequencing: 720-nt of the N-gene-encoding regions were amplified and the PCR products were purified with the QIAquick PCR Purification Kit, then sequenced with an ABI PRISM 3100 DNA sequencer.

Sequence analysis: Complete alignment of the nucleotide sequences was performed with the ClustalX program, v1.8. The MegAlign software (v5, DNASTar, Inc., Madison, WI, USA) was used to analyze the nucleotide and deduced amino acid sequence homologies. The neighbor-joining (NJ) method of MEGA3 v3.1 was used with 1000 bootstrap replications.

**Results:**

Specimen detection: 2887 dog specimens randomly collected in restaurants from Guizhou, Guangxi, and Hunan provinces between 2005 and 2007, from the DFA positive samples, 66 were positive for RABV RNA by RT-PCR, yielding a positive rate of 2.3%.

Sequencing and phylogenetic analysis: A stretch of 720 bp was obtained in a total of 60 specimens, all 60 sequences were submitted to GenBank (accession nos EF990564–EF990623). The 60 specimens were only from two kinds of hosts: dogs and humans. The human specimens showed

close relationships to the dog specimens from the same geographical area.

Most of the Chinese samples clustered together with other Asian dog-related RABV lineages, there was no close relationship between RABV from China and other south Asian countries, such as India and Sri Lanka. A component of the Chinese RABVs from Guizhou and Guangxi were related with the so-called cosmopolitan dog-related variant, including nearly all kinds of vaccine strains, and variants associated with skunks, wolfs, and red foxes. All the Chinese specimens were phylogenetically distant from the American bat-associated variants and lineages.

### **Discussion:**

Guizhou, Guangxi, and Hunan provinces have reported the highest incidence of human rabies in recent years and were selected for a national epidemic surveillance in 2005. According to the national data on rabies surveillance, domestic dogs are the major infection source to humans and other domestic animals.

Phylogenetic analysis here shows that the human specimens generally belong to the same branch as dog RABVs from the same geographical region. This finding provides virological evidence that domestic dogs are major reservoirs in human rabies epidemics. In addition, these results supported the inference that rabies viruses from same geographical regions tend to group together, representing the same rabies focus or same geographically circumscribed outbreak.

We observed an infection rate in dogs of 2.3% which comprised 15 cities in three provinces. The phylogenetic analysis of the specimens collected in these three provinces shows clearly that rabies viruses have a distinctive geographic structure. From an epidemiological perspective, these groups may be interpreted as currently ongoing independent dog rabies foci (or outbreaks).

Phylogenetic inferences together with epidemiological and historical data suggest that ongoing rabies foci in Guangxi and Guizhou provinces had its origins in Hunan province. This evidence reinforces the concept of dog rabies being moved from high incidence provinces. These findings suggest that the transprovincial spread of RABVs by the trade of dog meat may be one of the driving forces responsible for exacerbating the dog rabies epizootic, reflected by a human rabies

epidemic.

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- 3) Song M, Tang Q, Xu Z, Li H, Wang DM, Mo ZJ, et al. Analysis on the factors related to rabies epidemic in China in 2005. *Chinese Journal of Epidemiology.* 2006; 27:956-9.
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- 2) Du J, Zhang Q, Tang Q, Li H, Tao X, Morimoto K, Nadin-Davis SA, Liang G. Characterization of human rabies virus vaccine strain in China. *Virus Res.* 2008, Aug, 135(2):260-266.
- 3) "Molecular epidemiology of rabies in southern China" submitted to EID journal.

## PROJECT TITLE

### DEVELOPMENT, APPLICATION OF NEW METHODS FOR RABIES DIAGNOSIS IN NATIONAL INSTITUTE OF HYGIENE AND EPIDEMIOLOGY (NIHE), HANOI, VIETNAM

#### NAME OF RESEARCHERS

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#### SUMMARY

Rabies has been a serious public health problem in African and Asian countries including Vietnam. In recent years, Vietnam as well as the Philippine, Laos, Cambodia, and China are facing the problem of rapidly increasing human rabies cases. In Vietnam, 131 human rabies cases were reported in 2007 that might be the result of more focus on other infectious diseases such as SARS, Avian Influenza, and Cholera... The problem is that by now, *in Vietnam there is only one laboratory in the South for animal rabies diagnosis and only one laboratory for human rabies diagnosis in NIHE. No laboratory for animal, human rabies diagnosis exists in the remaining places such as in the North and Highland where there have been high incidence of rabies.* The National

rabies surveillance system focuses merely on clinical report, not confirmed by laboratory tests.

The symptoms of rabies are not specific in the early period of the illness, so it may be confused with other encephalitis diseases. That is why confirmation of rabies in the patients particularly in the early stage is very important, that leads to quarantine the patient and to prevent patient relatives and health care workers from the potential exposure to rabies virus. More importantly a human with potential exposure to rabies virus can be saved when rabies confirmation in animals can be achieved

The proposal of this project is to develop new and advanced methods for rabies diagnosis and apply them for rabies diagnosis in Vietnam.

## **PURPOSE**

1. To develop new and advanced techniques for rabies diagnosis and apply them for rabies surveillance in Vietnam.
2. To evaluate the technique established in NIHE
3. To apply new techniques for rabies diagnosis in Northern Vietnam

## **METHODS**

### ***1. Development of RT – LAMP and real-time RT – PCR***

- Primers for RT – LAMP and real-time RT – PCR were designed in collaboration with NIID based on N gene nucleotide sequences of 18 rabies viruses which were isolated from humans and dogs contracted rabies in Northern Vietnam.

## **2. *Evaluation of the new technique in NIHE***

- RT – LAMP, Real-time RT – PCR techniques originally developed were transferred to NIHE. The relevance of those methods for rabies diagnosis in Vietnam was validated using fixed rabies virus (CVS, Vnukovo-32) as well as samples from rabies suspected humans and animals
- Staff practical skills were evaluated by using positive and negative controls
- The effectiveness of new methods for rabies diagnosis was evaluated by comparison of the results with those obtained by RT – PCR, FA and virus isolation

## **3. *Application of the new techniques for rabies diagnosis in NIHE***

- Field visit to select the research area was conducted.
- Training of medical staffs of hospitals and district preventive medicine centers on bio-safety, sampling (intra vitam samples and animals brain operation), packing and transferring samples to NIHE was conducted.
- Pre – exposure prophylaxis was performed for staffs involved in this study

- Intra vitam samples of saliva (SLV) and cerebro-spinal fluid (CSF) were collected from rabies suspected humans in national hospitals which are located in the North of the country.
- Ammon's horn, cerebrum and cerebellum of sick dogs (Dogs have one of these symptoms: refuse or stop eating; saliva running; aggressive, paralyzes ...) from slaughterhouses in Hoai Duc were collected and analyzed by RT – PCR, FA and virus isolation

## RESULTS

### 1. *Development of RT – LAMP and real-time RT – PCR*

Primers for RT – LAMP and real time RT – PCR originally designed and developed in NIID were transferred to NIHE. The protocols for both methods were also developed under the collaboration between NIID and NIHE. The detail of the primers will be published elsewhere (Boldbaatar et al., in preparation).

The outline of RT-LAMP protocol developed is as bellow:

The RT-LAMP was carried out in a 25ul reaction volume using the Loopamp RNA amplification kit protocol (Eiken, Japan). The reaction mixture was consisted of 40pmol each of the FIP and BIP primers, 5pmol each of the F3 and B3 primers, 20pmol of the BLP primers, 1.0ul of enzyme mixture containing Avian myeloblastosis virus (AMV) reverse transcriptase and Bst DNA polymerase, 12.5ul of 2X reaction mixture

(40mM Tris-HCl, 20mM KCl, 16mM MgSO<sub>4</sub>, 20mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.2%Tween20, 1.6M Betaine, 2.8mM of each dNTPs) and 5ul of RNA sample. The mixture was incubated at 63<sup>0</sup>C for 1 hour and the reaction was stopped by heating at 80<sup>0</sup>C for 5 min. Then, RT-LAMP product was detected by the ethidium bromide staining and visualized under a UV transilluminator after 1.5% agarose gel electrophoresis with TAE buffer

## **2. Evaluation of the techniques in NIHE**

- It was confirmed that 3 staffs of the Rabies Lab in NIHE could perform those techniques.
- Primers for RT – LAMP and real-time PCR worked well with fixed rabies viruses (CVS and Vnukovo-32), but were not sensitive with all wild type rabies viruses isolated in Northern Vietnam except some

## **3. Results of rabies diagnosis**

### **3.1. Results of animal rabies investigation in Hoai Duc District, Hanoi**

#### **❖ Particular trait of Hoai Duc District**

- Hoai Duc is in delta region, and citizens mainly earn their living expense by agriculture, trade village and family handicraft.
- There are 20 communes and 119 hamlets in Hoai Duc district, in which the land area is about 82 km<sup>2</sup> and population size is 176,800.
- Domestic animals such as chicken, pigs, ducks, cattle and dogs are mainly reared by individual family

❖ *Dog vaccination*

- Dog raising is done only by individual family, and the number of dogs in each family is from 1-2 dogs in average
- There is one trade village, namely Duc Giang in Hoai Duc district. Their main job is dog butchering and supplying dog meat for Hanoi restaurants.
  - + There are 47 Dog slaughterhouses in Duc Giang trade village.
  - + In each commune, there are 1 – 3 dog meat restaurants, in which they butcher dogs and sell raw or cooked dog meat.
- Dog suppliers are mainly in Thanh Hoa province where dogs are imported from Thailand, Myanmar, Laos, and Cambodia. Dogs also are collected from individual families of surrounding districts and provinces. The places for investigation of rabies infection among sick dogs were selected and it was particularly focused on the slaughterhouses which gather dogs from surrounding areas

❖ *Summary of human and domestic animals vaccination against rabies from 2003 – 8/2008 in Hoai Duc District*

Year	Human vaccinated against rabies	Animals (dogs) vaccinated against rabies
2003	451	7,395
2004	350	7,087
2005	325	7,942

2006	317	7,022
2007	220	7,701
8/2008	113	6,820

### 3.2. Results of human rabies diagnosis

Intra vitam samples of 24 rabies suspected patients were tested by RT-PCR.

3.2.1. Detection ability of rabies virus RNA in intra vitam samples by direct RT – PCR

<i>Sort of samples</i>	<i>RT – PCR result</i>		<i>Total</i>
	Positive	Negative	
CSF	3	2	5
SLV	7	1	8
CSF + SLV	11	0	11
Total	21	3	24

When only CSF was available, 3 out of 5 samples tested positive (60%), while 7 out of 8 SLV were (88.9%) positive if SLV alone was submitted for the test. When both CSF and SLV could be taken from the patients 11 out of 11 patients were diagnosed as being infected with rabies virus (100%).

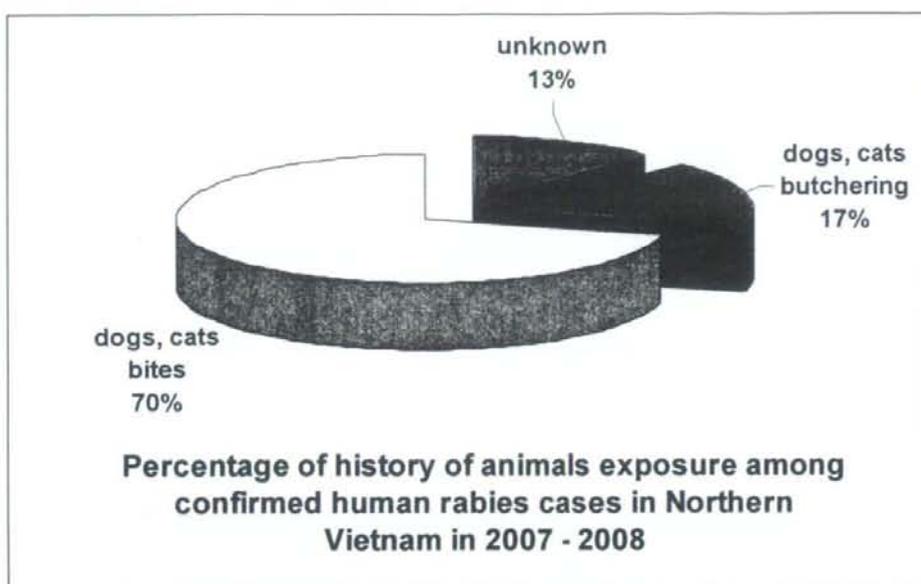
3.2.2. Period of sample collection after onset

The table shows the result of viral RNA detection by RT – PCR when samples taken at various times after onset of signs and symptoms. The result indicated that even shortly after the onset, RT- PCR could detect the presence of the virus. However, it seemed important to obtain the samples as early as possible, since the sensitivity of the detection appeared to decrease if samples were taken later after the onset of the disease.

<i>Collection time</i>		<i>1<sup>st</sup></i>	<i>2<sup>nd</sup></i>	<i>3<sup>rd</sup></i>	<i>4<sup>th</sup></i>	<i>&gt;5<sup>th</sup></i>	<i>Total</i>
PCR results	Positive	6	4	5	2	4	21
	Negative	0	0	1	0	2	3
Total		6	4	6	2	6	24

### 3.2.3. History of exposure to suspected rabid animals among confirmed patients

We have then analyzed as to whether the patients had any known contacts with animals. The result is shown in the figure.



In this study 13% of human rabies cases did not have any history of dog or cat bites; 17% had butchered sick dogs and cats, and 70% were bitten by dogs or cats.

### 3.3. Results of rabies confirmation in sick dogs in Hatay slaughterhouses

The brains of 100 sick dogs were collected from the slaughterhouses in Hoai Duc district and brain samples were examined for the presence of rabies virus by RT – PCR.

Two out of 100 sick dogs were confirmed to be infected with rabies virus. The rate of rabies infection among sick dogs in the slaughterhouses in Hoai Duc district, Hanoi, therefore was found to be 2% in this study

#### *Results of laboratory diagnosis among 100 sick dogs in slaughterhouses*

<i>Techniques</i>	<i>Positive</i>	<i>Negative</i>	<i>Total</i>
<b>FA</b>	0	100	100
<b>RT – PCR</b>	2	98	100

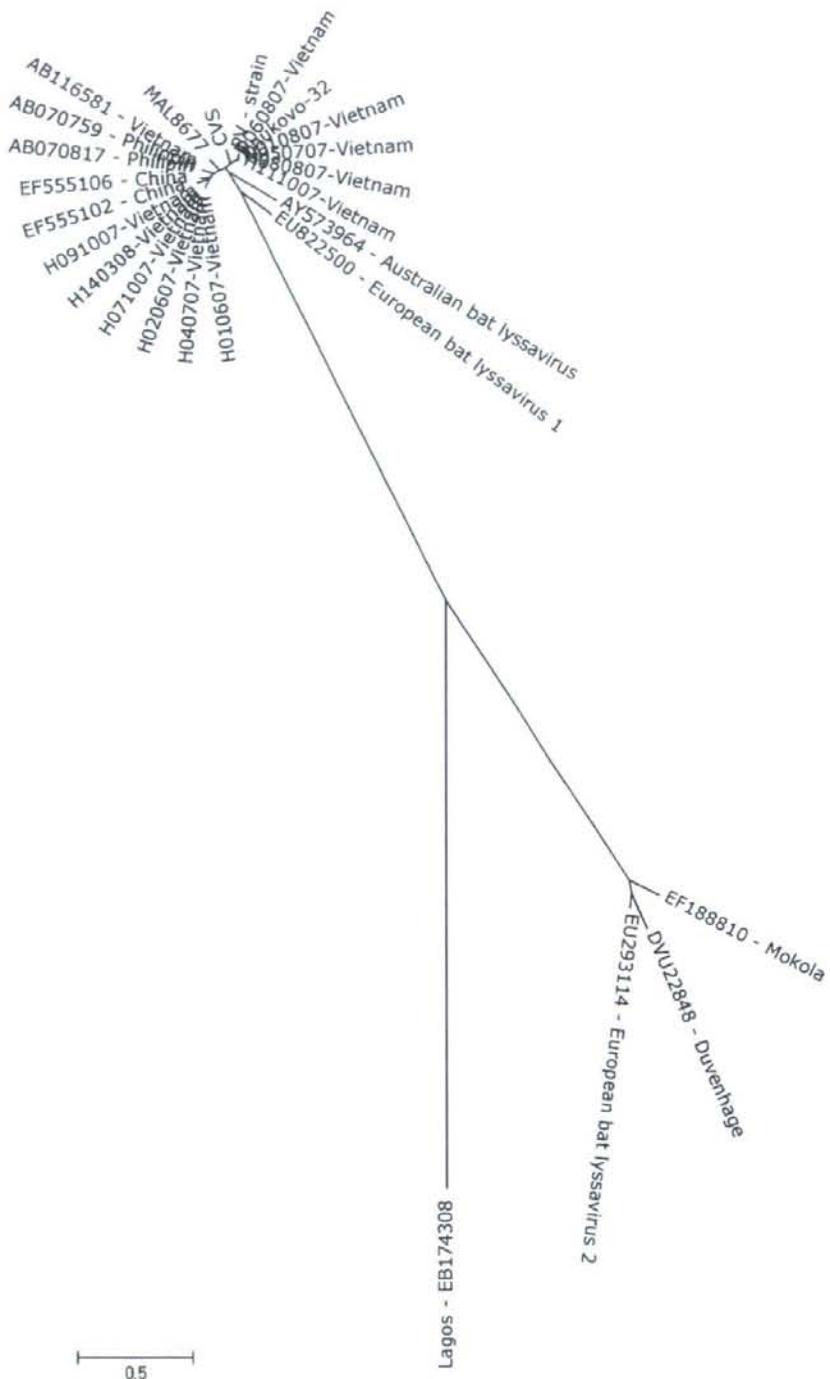
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<b>Virus isolation</b>	0	100	100
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*3.4 Phylogenetic tree of wild type rabies viruses isolated in Northern Vietnam in 2008*

We have then sequenced the amplicons obtained by RT-PCR and phylogenetic analysis was conducted. The result is shown below.



## DISCUSSION

- ◇ It is essential to improve the specificity and sensitivity of primers for RT – LAMP and RT – real time PCR. The improvements of those primers will be feasible, because N gene nucleotide sequence of wild type rabies viruses which are circulating in Vietnam in recent years can be determined.
- ◇ It is necessary to standardize new techniques developed under the collaboration with NIID and then to evaluate the effectiveness of those techniques (sensitivity and specificity) in comparison with other basic techniques such as FA, virus isolation and RT - PCR
- ◇ The advantages of RT – LAMP technique are that there is no requirement of expensive equipments such as PCR machines or a system for electrophoresis. So it seems suitable to apply this technique for rabies diagnosis in most of hospitals and or laboratories in Vietnam which are not well equipped
- ◇ 17% (4 patients) of confirmed rabies in humans had history of butchering sick dogs and cats. To better understand this issue, we conducted the study of 100 sick dogs in slaughterhouses and confirmed that the rate of rabies infection was 2% among those dogs. This evidence showed that one of the human rabies transmission route in Vietnam which was not well reported in the world is butchering rabid animals

- ◇ 13% (3 patients) of confirmed rabies in humans had neither history of dog bites nor butchering dogs or cats. Hypothesis is eating dog blood pudding leads to rabies infection?

**Publication list for this work**

1. Nguyen thi Kieu Anh, Ngo Chau Giang, Nguyen Vinh Dong. Quick detection of genomic rabies virus by direct RT – PCR, Journal of Military Pharmacy – medicine, volume 33, NO 2/2008
2. Nguyen Thi Kieu Anh, Nguyen Thi Hong Hanh, Ngo Chau Giang, Nguyen Vinh Dong, Phan Tien Tan, Nguyen Quoc Thai, Pham Hong Nhung. The preliminary human rabies cases confirmed by laboratory diagnosis and prototype study on the risk of rabies infection by exposure with agesta of rabid animals, presentation in conference of rabies in America, Oct, 2008 CDC, USA

**Title: Construction of laboratory network on the molecular epidemiology of Rabies and the development and standardization of Rabies diagnostic methods**

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## Summary

Strengthening of rabies diagnostic laboratories has been recognized by the Philippine National Rabies Prevention and Control Program as a strategic approach to elimination. In the Philippines, rabies diagnosis using the fluorescent antibody test (FAT) is performed by the Regional Animal Disease Diagnostic Laboratories (RADDL) under the Department of Agriculture. This study was conducted to develop and standardize rabies diagnostic methods in the Philippines and to establish a laboratory network for the molecular epidemiology of rabies in the country.

The first component of the study involved the development and standardization of rabies diagnostic methods. Polyclonal antibodies produced by NIID, Japan were used in the optimization of the Modified Direct Rapid Immunohistochemistry Test (DRIT) at RITM. Initial results show that there was no marked difference in the DRIT and FAT results of eight dog brain tissue samples using 1:1000 dilution of the 70 ug/ml antibody.

The second component of the project involved the establishment of a laboratory network to conduct molecular epidemiology studies of rabies in the Philippines. This network included 4 laboratories in the regions: RADDL 3, RADDL 5, RADDL 7 and RADDL 10. The four laboratories and their respective technicians were assessed in their performance of rabies diagnosis using FAT. Thereafter, the laboratories submitted brain tissue samples for confirmatory FAT and reverse transcription polymerase chain reaction (RT-PCR) at the RITM. Confirmatory FAT and RT-PCR on the rabies glycoprotein (G) gene was already done on the 75 FAT-positive samples submitted as of December 2008, 52 of which were already sequenced. Phylogenetic tree of the 52 samples suggests that there are different strains persisting in each Region.