Table 1. Diagnostic results of specimens randomly collected from domestic apparently "healthy dogs"

Cities	Number of specimens	Number of positives	Positive rate (%)
Guizhou province			
Qianxinan (QX)	427	13	3
Qiannan (QN)	54	1	1.9
Anshun (AS)	173	8	4.6
Liupanshui (LP)	200	0	0
Guangxi province			
Laibin (LB)	213	4	1.9
Guigang (GG)	304	8	2.6
Hechi (HC)	206	1	0.4
Yulin (YL)	302	4	1.3
Nanning (NN)	222	7	3.2
Liuzhou (LZ)	105	2	1.9
Hunan province			
Shaoyang (SY)	151	6	4
Yongzhou (YZ)	162	2	1.2
Xiangtan (XT)	114	3	2.6
Changde (CD)	102	4	3.9
Xiangxi (XX)	152	3	1.9
Total	2887	66	2.3

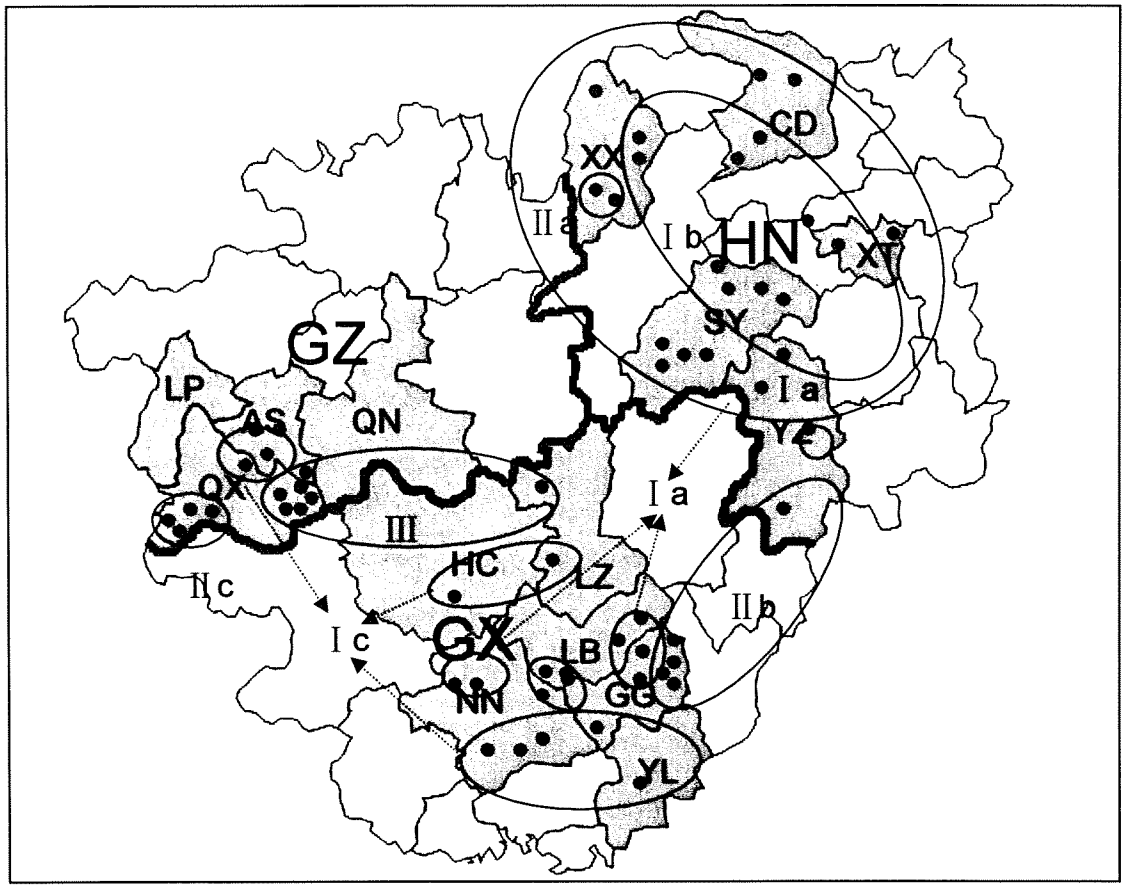

Figure 4. The geographic distribution of the 15 cities selected for specimen collection in Guizhou (GZ), Guangxi (GX), and Hunan (HN) provinces, and of genetic groups and subgroups of the 60 samples. Roman numbers and letters indicate genotypes, gray areas indicate regions selected for specimen collection, circles indicate specimens collected, ovals indicate regions with the same genotype, and arrows indicate specimens with the same genotype. LP, Liupansui; AS, Ansun; QN, Qiannan; QX,Qianxinan; XX,Xiangxi; CD, Changde;XT, Xiangtan; SY,Shaoyang; YZ, Ynagzhou; HC, Hechi; LZ, Liuzhou; LB, Labin; GG, Guigang; NN, Nanning; YL, Yulin.

A stretch of 720 bp of the N gene of the RABV-positive specimens was obtained for a total of 60 specimens. These comprised the following: 53 were from the 66 specimens collected from domestic apparently "healthy dogs", and 7 were from the 4 rabid dogs and 3 patients. The remaining 13 positive specimens could not be successfully sequenced. All 60 sequences were submitted to GenBank (accession Nos EF990564–EF990623). The phylogenetic analysis of the specimens collected in these three provinces(Fig 5) shows clearly that rabies viruses have a distinctive geographic correlation, where Group I predominantly encompassed Hunan, Group II, Guangxi and Group III, Guizhou. From an epidemiological perspective, these groups may be interpreted as currently ongoing independent dog rabies foci (or outbreaks) (Molecular Epidemiology of Rabies in Southern People's Republic of China. Emerging Infectious Diseases• 2009, 15(8)1192-1198.).

The analysis represented the phylogenetic relationship and geographic distribution characteristics of rabies virus in southern China showed that the spread of rabies viruses from high-incidence regions, particularly by the long-distance movement or transprovincial translocation of dogs caused by human-related activities, may be one of the important causes for the recent massive human rabies epidemics in southern China (Molecular Epidemiology of Rabies in Southern People's Republic of China. Emerging Infectious Diseases• 2009, 15(8)1192-1198.).

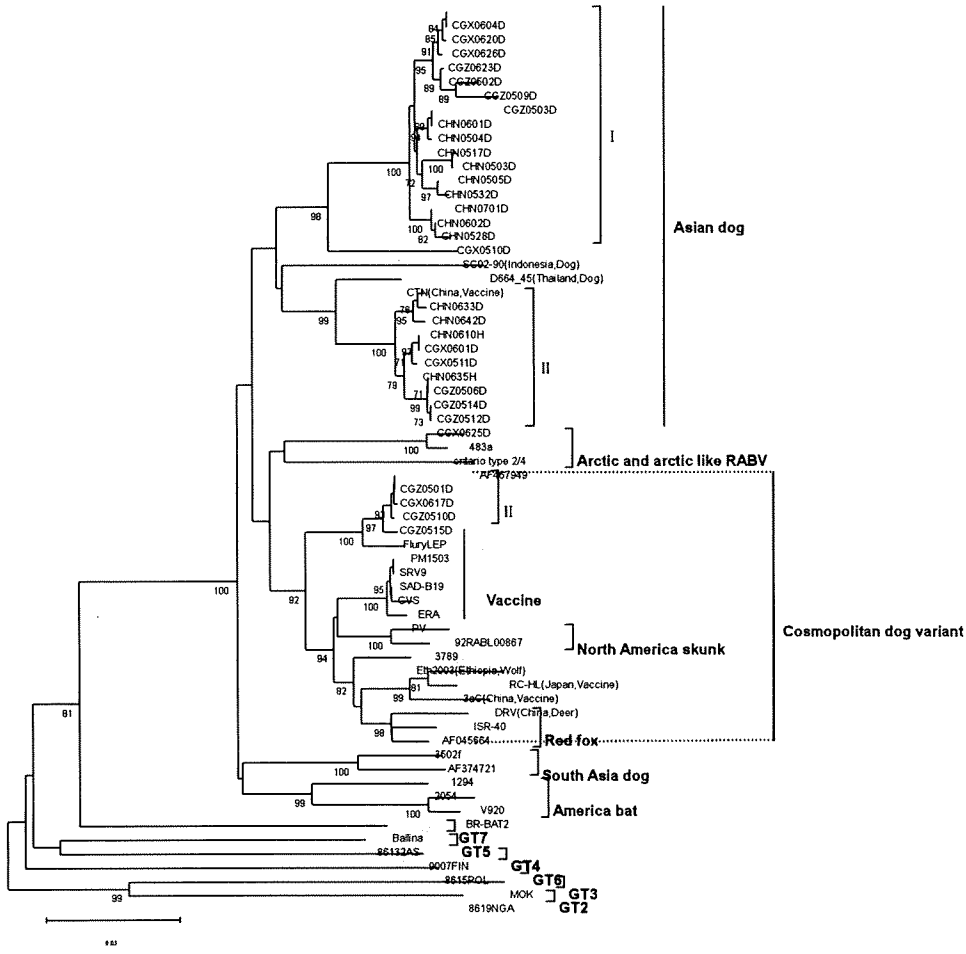

Figure 5. Neighbor-joining phylogenetic tree of 60 specimens from China and different genotypes (GT) throughout the world, based on the N fragments 720-nt long (nt 634–1353) of the rabies virus. The number at each node indicates the degree of bootstrap support, although only those with support greater than 70% are indicated. (Molecular Epidemiology of Rabies in Southern People's Republic of China. Emerging Infectious Diseases• 2009, 15(8)1192-1198.)

The present state of the infrastructure of diagnosis in China.

According to the National Plan of Rabies Surveillance in China, provincial CDCs should be responsible for collection and summarization of results of laboratory rabies

examinations in areas under its jurisdiction, and input all data of this quarter into rabies surveillance database at the end of each quarter, and feed them back to each surveillance sites at the end of each quarter. The laboratory of National Institute for Viral Disease Control and Prevention in China CDC should feed identification results back to provincial CDCs within one month upon specimen reception.

Laboratory detections for rabies conformation in China include the techniques suggested by WHO Expert Consultation on Rabies 2005, which are as the follows:
(1) The direct fluorescence assay (DFA) technique for antigen detection: prepare smears of saliva, nasopharyngeal washing or cerebrospinal fluid within the first week of onset, corneal imprints, skin sections, stain with fluorescent antibody against rabies, positive for rabies virus antigen detection
(2) Viral nucleic acid detection by RT-PCR: obtain saliva, nasopharyngeal washing, cerebrospinal fluid cornea, or skin tissue within the first week of onset, detect by RT-PRC, positive for viral nucleic acid detection
(3) Collect postmortem brain tissue, positive for viral isolation or fluorescent antibody staining, viral nucleic acid detection by RT-PCR
Specimens with positive results in viral antigen and nucleic acid detection are used for nucleic acid sequencing and analysis.

The results and the continuing work.

To improve the capacity of rabies laboratory surveillance in China
The national training workshop hand by hand on rabies laboratory detection techniques was organized for the staffs from the provincial CDCs in October 2009 in Nanning city of Guangxi province, over 80 participants from 30 provinces/autonomous regions or cities' CDCs or veterinary departments come to Nanning city to take part in this training workshop, the training courses include: updates the national rabies surveillance system, laboratory hands-on training on testing methods such as the DFA technique for antigen detection, the rapid fluorescent inhibition test (RFFIT) of rabies virus neutralizing antibody and RT-PCR method.

To establish the rapid fluorescent inhibition test (RFFIT) of rabies virus neutralizing antibody for the surveillance of rabies post-exposure prophylaxis and treatment
In the laboratory of National Institute for Viral Disease Control and Prevention in China CDC: CVS-11 was used for the standard challenge virus and prepared three generations and established the virus library. Used international standard for rabies immunoglobulin as the reference serum and established the rapid fluorescent inhibition test using the protocol of Institute of Pasteur, and validated the specificity, stability and reproducibility. We successfully established the rapid fluorescent inhibition test and the test had good specificity stability and reproducibility. The establishment of the rapid fluorescent inhibition test has important effect for the monitoring of rabies and has been used on the surveillance of rabies post-exposure prophylaxis and treatment.

Primary surveillance of rabies post-exposure prophylaxis by RFFIT

213 human serum samples are tested in parallel by RFFIT between institute for viral disease control and prevention, Chinese center for disease control and prevention (IVDC, CCDC) and national institute for the control of pharmaceutical and biological products (NICPBP). The genomic means tiltration (GMT) is 1.90 IU/ml and 1.81 IU/ml without statistic difference. RFFIT results for most samples with rabies virus neutralizing antibody (RVNAs) between 1 and 25 IU/ml varied in a narrow range, but for most samples with RVNAs over 25 IU/ml varied in a relative large scale. All results indicate the equivalent efficiency for RFFIT systems in two labs.

Expanding the etiological studies of rabies in China

Rabies epidemic areas were expanded from the south part into the north-western part of China following the continuing increasing of rabies incidence in China, at the same time, several new wild animal host were found and identified through rabies antigen detection and virus isolation, which means that there existed more infectious origins for human rabies in China except dogs and cats.

To expand the etiological studies of rabies in China is necessary, for this purpose, we select three highest epidemic provinces (Guangxi, Guizhou and Hunan), the medium epidemic districts (Anhui, Zhejiang, Jiangsu and Shandong provinces) as well as the low risk districts (Yunnan province and Shanghai city) as the areas of the etiological studies of rabies based on the three highest epidemic provinces investigation. Brain specimens from animals and suspected patients were collected at the districts of high-, medium- and low incidence rates of human rabies and detected by both DFA and RT-PCR. 254 of 3007 specimens of dog brains were positive by DFA (positive rate of 8.4%), 78 of these 254 samples were positive by RT-PCR(positive rate of 30.7%); 63 of 93 specimens of dogs and cats attacking humans were positive by DFA(positive rate of 67.7%)and all the 63 specimens were positive by RT-PCR. In addition, RV could also be detected in Apodemus agrarius, ferret badger, and suspected patients specimens from survey district. There was no statistical difference between the infection rates of rabies virus in different province and in regions with different incidence of rabies of each province. There could be a relative high infection rate of rabies virus among domestic dogs (cats) in endemic areas in China and wild animals could be infected with rabies virus in the survey district.

The sequencing and molecular characterization analyses of these new specimens are continuing going on.

YEAR 2 PROJECT REPORT

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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OBJECTIVES

1. To establish and evaluate RT – LAMP and Real time PCR techniques for rabies diagnosis in NIHE
2. To analyze molecular epidemiology of rabies virus circulating in Northern Vietnam in recent years

PART 1: SUMMARY OF RABIES SITUATION IN VIETNAM

1. Human rabies epidemiology in Vietnam

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, from 2007 to 2009 nearly 300 human rabies cases were reported and rabies epidemic occurred in 25 – 27 provinces (chart 1 and 2).

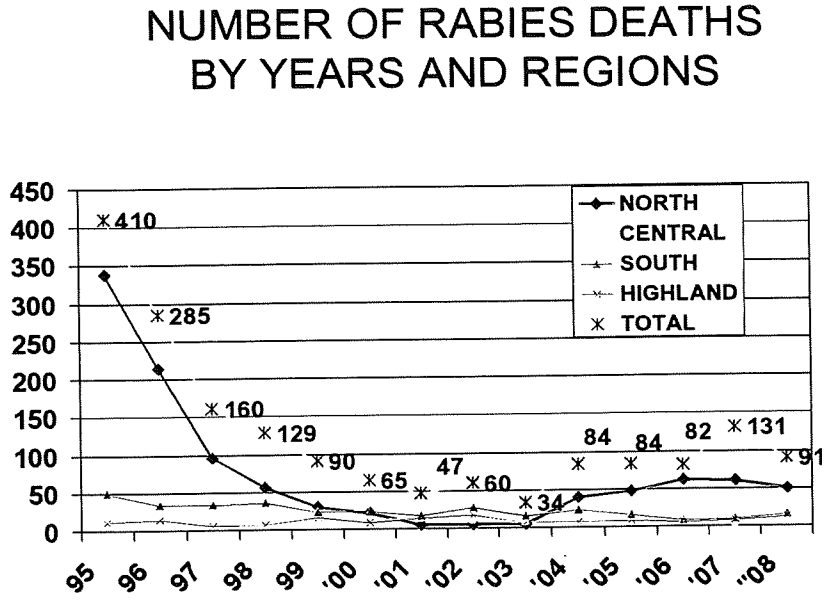


Chart 1: Number of rabies deaths by regions (1995 – 2009)

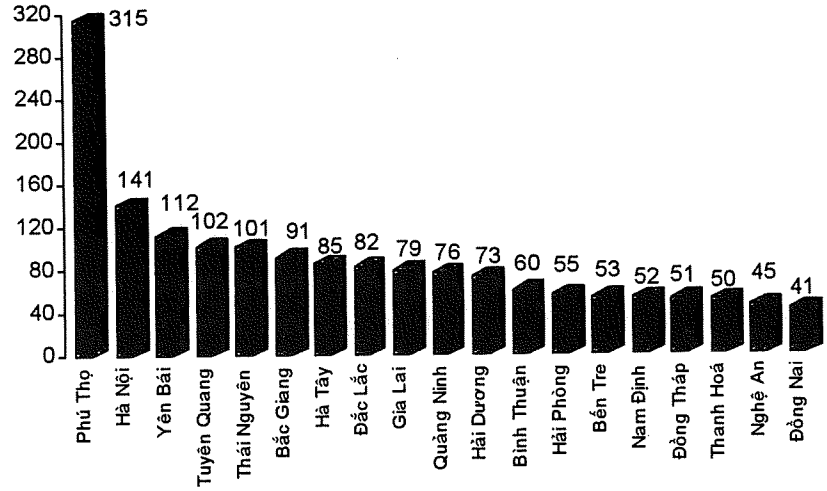
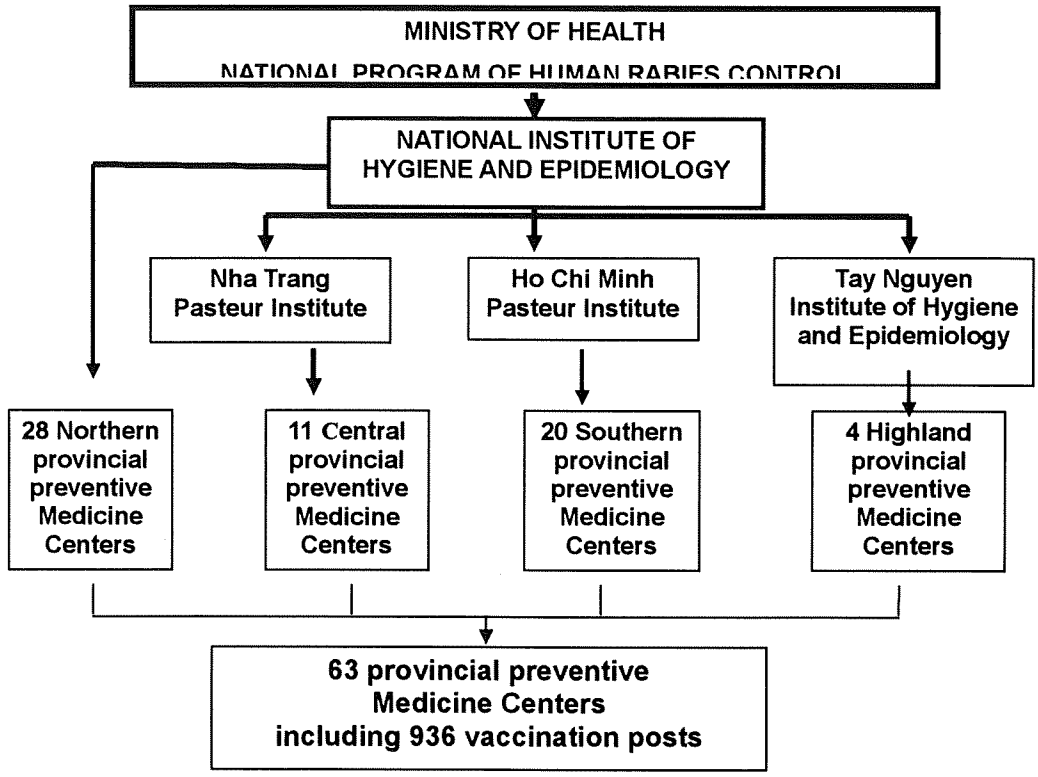


Chart 2: High rabies human deaths provinces in the period 1994 - 2009

2. System for rabies surveillance, control and prevention in Vietnam

We do not have the system for rabies surveillance in animals, but the system for human rabies surveillance is available and belongs to Ministry of Health (diagram 1). The annual data of human deaths due to rabies reported by this system based on the clinical diagnosis, not by laboratory confirmation. Therefore, to better surveillance and control of rabies, it is important to establish a national program for rabies control and prevention including intervention and cooperation between animal and human sites combine with laboratory network for rabies diagnosis.

Diagram 1: System for human rabies surveillance in Vietnam



3. Laboratory network for rabies diagnosis and surveillance in Vietnam

There is no laboratory network for rabies diagnosis in Vietnam. Only two laboratories for rabies diagnosis are available, one laboratory for rabies diagnosis and research in the North (NIHE) and one laboratory for animal rabies diagnosis in the south (Center for animal diagnosis and treatment in Ho Chi Minh city).

The role of rabies laboratory in NIHE: Rabies laboratory at NIHE belongs to the Virology department. It started running from 2007, at first we developed and applied the molecular biology techniques to diagnose rabies suspected human who admitted to Bach Mai hospital and the National Institute for Tropical and Infectious Diseases (both hospitals are located in Hanoi). From 2007 – 2009, the rabies laboratory at NIHE had been supported by Ministry of Health to implement the Ministry level project ***"Research on rabies virus molecular epidemiology in Vietnam"*** and from 2008, it was supported by NIID to carry out the project namely ***"Development, application of new methods for rabies diagnosis in NIHE"***. All the activities of rabies laboratory were supported financially by these two projects and are to carry out researches to fit the objectives of these two projects only. By now, rabies laboratory at NIHE has not been responsible for rabies diagnosis in human rabies surveillance system that NIHE is the technical leader.

The role of rabies laboratory in Ho Chi Minh city: This laboratory has been supported by Ho Chi Minh People's Committee since 1996 to bear for animal (dogs, cats) rabies surveillance in Ho Chi Minh city **only**. Table 1 showed the data of annual rabies surveillance among dogs in Ho Chi Minh city.

Table 1: Rabies laboratory surveillance among dogs in HCM city

Year	Samples	Positive (FAT)	
		Numbers	(%)
1996	69	41	59.4
1997	57	26	45.6
1998	73	18	24.6
1999	61	03	4,92
2000	50	06	12.0
2001	40	03	7.5
2002	55	02	3,64
2003	24	00	0
2004	31	02	6.5
2005	20	00	0
2006	18	03	16.7
2007	19	02	10.5
2008	12	00	0

Process of establishing rabies laboratory network

Three regional laboratories in three provincial preventive medicine centers were set up for animal sample collection. They are located in the north (Ha Tay province), highland (Gia Lai province) and the center of Vietnam (Binh Thuan province). Three laboratories were equipped with the essential tools, facilities for animal operation, and all staff of those laboratories were vaccinated and trained on bio-safety, techniques for operating animals, taking, packing and shipping samples. Since 2008, those laboratories have started operations to take samples

of rabies suspected animals in the regions, by now 176 dog samples have been collected and analyzed.

Rabies laboratory at NIHE then cooperated with JICA to develop SOP for bio-safety laboratory as well as SOPs for rabies diagnosis techniques, including the SOP for collection, transportation, shipment and storage of samples. Now, this SOP is developing, editing and auditing with the technical assistance of NIID experts.

PART 2: DEVELOPMENT OF RT-LAMP IN VIETNAM

1. Materials and methods

* *Primer design*: Primers for RT – LAMP were designed in collaboration with NIID based on partial N gene nucleotide sequences of 18 rabies viruses which were isolated from humans and dogs contracted rabies in Northern Vietnam.
* *Protocol development*: based on the protocol of RT – LAMP kit
* *Evaluation of the new technique in NIHE:*
  - RT – LAMP techniques originally developed was transferred to NIHE. The relevance of this method 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

* *Application of the new techniques for rabies diagnosis in NIHE*
  - 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, paralyses ...) from slaughterhouses in Hoai Duc were collected and analyzed by rt – LAMP, RT – PCR, FA and virus isolation

2. Results

2.1. The RT – LAMP technique was developed as bellow:

❖ ***Primers designed for RT – LAMP:*** Primers were designed based on the partial nucleotide sequence of N gene of 18 rabies strains isolated in Vietnam. Eleven primers for RT – LAMP were designed and shipped to the rabies laboratory at NIHE in 2009. Table 2 shows the name and nucleotide sequence of primers for Vietnam RT – LAMP.

Table 2: 11 primers for RT- LAMP to detect Vietnam rabies strains

No	Primers	Nucleotide sequence
1	Viet F3-1	ACA TGT CCG GAA GACT
2	Viet F3-2	ACC TGC CCT GAG GAC T
3	Viet FIP-1	ACC AGA GAA CCA GGG GTG AGG ACC AGC

		TAT GGA ATC
4	Viet FIP-2	ACA AGA GAA TCA GGG GTG AGG ACC AGT TAT GGA ATC
5	Viet FIP-3	ACA AGA GAA TCA GGG GTG AGG ACC AGT TAT GGA AT
6	Viet BIP-1	AAG GGA ATT GGG CTC TGA CCT AAG GAC GCA TGC TCA G
7	Viet BIP-2	AAG GGA ATT GGG CTT TGA CCT AAG GAT GCA TGC TCA G
8	Viet BLP-1	GGC ATG GAA CTG ACA AGA GAC C
9	Viet BLP-2	GGT ATG GAG TTG ACG AGG GAC C
10	Viet B3-1	CAG ACT CAA GAG AAG ACC
11	Viet B3-2	CAG ACT TAA GAG AAG CCC

❖ ***The prototype SOP for RT – LAMP was set up 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-HCI, 20mM KCI, 16mM $MgSO_4$, 20mM $(NH_4)_2SO_4$, 0.2%Tween20, 1.6M Betaine, 2.8mM of each dNTPs) and 5ul of RNA sample. The mixture was incubated at 63^0C for 1 hour and the reaction was stopped by heating at 80^0C 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.

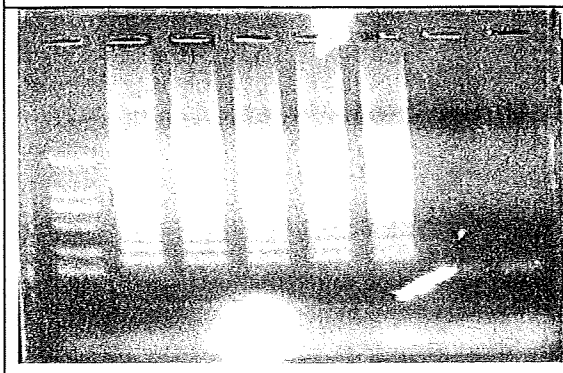
2.2. Evaluation of RT – LAMP and primers used for this technique

We used the laboratory rabies virus strain (Vnukovo-32) as the template to assess the sensitivity of RT – LAMP and its primers. Eleven primers were respectively replaced to form 19 primer sets (please see the index). The results show that the primer sets 1, 4, 5, 11, 14 and 17 are the most sensitive with Vnukovo-32 (table 3), the limit of RT - LAMP detection when using those primers is at 10^{-4} RNA dilution, equal to 2x10 RNA molecules (Figure 1). Only primer set 5 has the limit of detection at 10^{-5}, equal to 2 RNA molecules.

Table 3: The primer sets are the most sensitivity among the primer designed for Vietnam RT – LAMP

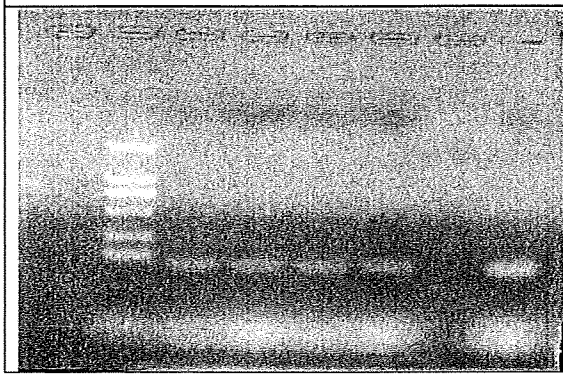
Primer set No	Primers
Set 1	F3 – 1; B3 – 1; FIP 1; BIP -1; BLP 1
Set 4	F3 – 2; B3 – 2; FIP 1; BIP -1; BLP 1
Set 5 (the most sensitive)	F3 – 1; B3 – 1; FIP 1; FIP -2; BLP1 and BLP 1 **(6 primers)**
Set 11	F3-2; B3-2; FIP-1; BIP-1; BLP-1; and BLP-2 **(6 primers)**
Set 14	F3 – 1; B3 – 1; FIP 1; BIP -2; BLP 1
Set 17	F3 – 2; B3 – 2; FIP 1; BIP -2; BLP 1

Figure 1: The assessment of primer sensitivity

Eletrophoresis result	Sample loading scheme
	1: Marker 2: $2x10^5$RNA 3: 10^{-1} dilution 4: 10^{-2} 5: 10^{-3} 6: 10^{-4} 7: 10^{-5} 8: Negative control

Two primer sets which did not anneal with Vnukovo-32 RNA orcDNA were shown in table 4

Table 4: The primer sets did not anneal with Vnukovo-32

Primer set No	Primers
Set 2	F3 – 1; B3 – 1; FIP 2; BIP -2; BLP 2
Set 3	F3 – 2; B3 – 2; FIP 2; BIP -2; BLP 2
Eletrophoresis result	**Sample loading scheme**
	1: Marker 2: $2x10^5$RNA 3: 10^{-1} dilution 4: 10^{-2} 5: 10^{-3} 6: no sample 7: Negative control

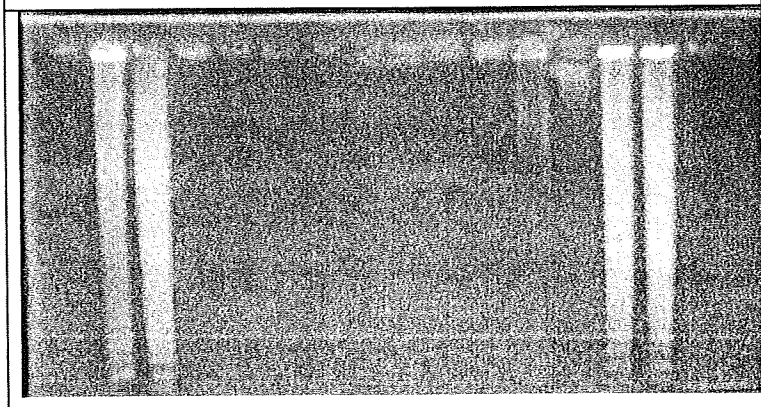
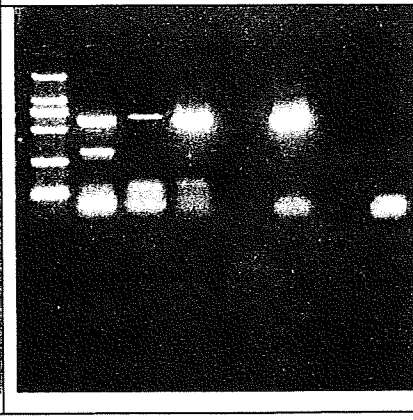
2.3. Effectiveness of RT – LAMP for rabies diagnosis in Vietnam

The positive human samples (saliva - SLV and, or cerebro-spinal fluid - CSF) which were detected by RT - PCR were used to evaluate the effectiveness and the sensitivity of primer sets as well as the RT – LAMP technique.

Table 5: Results of RT – LAMP diagnosis of positive human samples

Primer set	RT – LAMP		RT – PCR positive samples	Positive percentage of RT - LAMP
	Positive	Negative		
Primer set No 5	0	5	5	0
Primer set No 1	3	8	11	27.3%
11 primers	2	6	8	25%
11 primers	5	5	10	50%

Following the prototype SOP, the sensitivity of RT – LAMP was from 0 – 27.3% depending on the primer sets used, therefore we tried to modify the SOP by using all primers (11 primers) for the RT – LAMP reaction mixture and concurrently, increase the reaction and sample volumes to 50µl and 10µl, respectively. With this modified SOP, the sensitivity of RT – LAMP seemed to be higher (50%), it could detect seven among eighteen positive human samples detected by RT – PCR. All positive samples confirmed by RT – LAMP were very strong positive with RT – PCR (ex: sample number 20 and 35 presented in the figure 2a and 2b). None weak positive samples detected by RT – PCR could be determined by RT – LAMP (ex: sample number 02).

Fig. 2a: Electrophoresis of RT – LAMP products	Fig. 2b: RT – PCR products
M 20 PC 02 35 35 NC	M 20 02 35 PC NC
	

3. Conclusion: It needs to find out the optimal condition for the RT – LAMP as well as the most suitable, sensitive primers for Vietnam rabies wild type strains detection.

PART 3: RABIES DIAGNOSIS AND MOLECULAR EPIDEMIOLOGY OF RABIES VIRUS IN VIETNAM

1. Materials

Samples:

- 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. Those samples were confirmed infection to rabies virus by RNA detection using RT – PCR method

- Ammon's horn, cerebrum and cerebellum of sick dogs (Dogs have one of these symptoms: refuse or stop eating; saliva running; aggressive, paralyses ...) from slaughterhouses in Northern provinces
- Rabies suspected dogs in Center, highland and Southern provinces were collected and sent to rabies laboratory at NIHE
- The samples which were obtained from animals were analyzed by three methods, those are RT – PCR, FA and virus isolation on NA cell line.

Reagents and kits:

- Rneasy Lipid Tissue (QIAGEN)
- RNA viral purification from plasma and serum kit (QIAGEN)
- One Step RT – PCR (QIAGEN)
- Bigdye (AB)
- Dyex (QIAGEN)
- Agarose
- TAE buffer
- Ethidium Bromide
- Mab anti Rabies Nucleoprotein - FITC (Biorad)
- Slides and 96 well culture plates (Nunc)
- N7 (15 – 34) and JW6E (601 – 619) targeted to N rabies virus genome

Facilities and equipments

- BSL3 laboratory was used for samples treatment and virus isolation
- UV microscopy
- PCR and 3100 sequencer
- Bioedit, laser gene and Mega 4 softwares to clean up the data and construct phylogenetic tree
- Reference nucleotide sequences of N gene of rabies viruses achieved at www.ncbi.nlm.nih.gov. Those are (X03673 - PV; D42112- CVS; X13357 - PM; U2477 – ME9126; U22479- Brasil; U22627 – Egyp 8692; U22637 – Eth 8807; U22641 – Gui 9024; U22633 – AFS 8821; U22656 – RUS 9141; U22747 –Fran 9147; U22840 – Pol 8618; U22917 – SRL 94257; U22918 – Nep94260; EF 555106- China; EF555102- China; AB 070817 - Phi; AB070759- Phi; AB11658-Viet) and U22916-Mal 8677).

2. Methods

❖ **FAT: was performed by rabies laboratory SOP**

❖ **RT – PCR: the SOP as follow**

Mixture:

DW:	20 µl
dNTPs:	2 µl
5x Buffer:	10 µl
Backward primer (JW6E – 10 µM):	3 µl
Forward primer (N7 – 10 µM):	3 µl
Enzym mix:	2 µl

RNA:	10 µl
Total :	50 µl

Temperature cycles:

50°C:	30 min	
95°C:	15 min	
94°C:	1 min	35 cycles
54°C:	1 min	
72°C:	1,5 min	
72°C:	10 min	
4°C:		

Sequencing: Direct sequence method

PCR products were purified by gel extraction or colum. Purity DNAs were then amplified for sequence using forward and backward primers.

Following, PCR sequencing products were purified by dyex kit and annalized by 3100 sequencer.

Phylogenetic tree construction:

Cleaning up the nucleotide sequence by bio-edit and laser gene softwares. The phylogenetic tree was constructed by Mega 4 software.