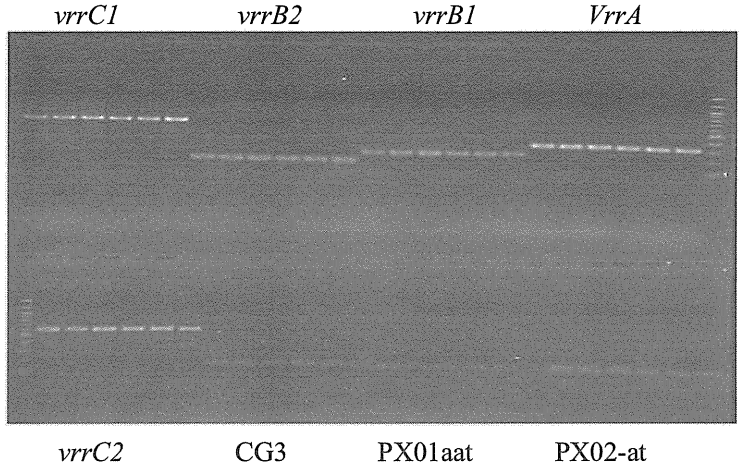
BA6.12	DienBien	vesicular fluid	4	20	8	57	21	1	7	7

Fig 1. PCR reaction of each primer of marker locus



In this time, all animal samples and soil samples were negative with both culture and PCR.

6.10 Discussion

Nowadays, anthrax is still considered one of the most dangerous zoonotic infectious diseases and especially, the agent has been used for biological weapon. Therefore, molecular epidemiological studies with adequate monitoring and surveillance for anthrax are essential to prevent the outbreak disease and minimize its threat.

In the published studies, PCRs with the specific nucleic acid probe for *B.athracis* (no cross-reactivity with other bacterial specific) have been reported. The primers often use to amplify the target genes such as *pag, cag, lef*...that are on plasmid pXO1 and pXO2. In our study, PCR using the specific primers (PA7/6 and MO11/12) has determined *B.anthracis* virulence genes from the clinical samples while culture result was negative. That means the bacteria might be died because of transportation time. Therefore, PCR is very useful for identification of agent in the high mountain areas in Vietnam.

On the other hand, the DNA fragments can be analyzed by multiple-locus variable number tardem repeat to describe molecular characterization of obtained strains and compared with *B.anthracis* strains in different time and areas. The MLVA-8 results showed that, six *B.antracis* isolates from different time and areas are the same cluster and number or repeats in the locus (Table 3 and Fig.1). However, MLVA 8, based on only 8 locus data, and its results greatly depend on the numbers and kinds of strains used for analysis. Therefore, we should used 80SNPs that already used in NIID. In the third year, we consider that full genome sequencing of some Vietnamese isolates to identify Vietnamese specific SNPs should be done. These SNPs would be the powerful and appropriate typing tool of your local isolates.

These results suggest that the genetic sequence of the *B.anthracis* strains were conserved; and no genetic differences associated with the geographical distribution and time of six strains. Among them, however, three strains were isolated from the patients who didn't contact with infected animal. In such cases, this leads to the question what is the real source of the infection. Environmental sample such as soil can be important source and route of anthrax infection in Vietnam.

As literature, it is very difficult to directly detect *B.anthracis* from soil samples by both culture and PCR. There are many contaminated and inhibitor factors in the soil samples. In the last period study, we were successful to apply a conventional PCR using Ba813R1/R2 primer to detect *B.athracis* spore in the artificial soil samples. However, the result was based

on the experimental stage. Up to now, there is still lacking evidence of isolated *B.anthracis* strain from soil samples in Vietnam. Therefore, it is necessary to find out a linked between animal – human- environment by using modern molecular methods. Further molecular epidemiological studies need to be done to establish etiology of *B.anthracis* and initiate strategies to implement anthrax control measure in Vietnam.

6.11 Publications

Poster day at the Southeast Asian One Health University Network (SEAOHUN) Executive Board Meeting in Bali, Indonesia 1-2 December 2012. Supported by RESPOND, USAID
Title: ***Anthrax report case in Vietnam: An update***

6.12 Reference

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We would like to thank the International Cooperation Department, NIHE for administration support.

Research 7

7.1. Project title: ***Enhancement of NIHE rabies laboratory capacity for rabies/bat lyssavirus diagnosis and research.***

7.2. General objectives:
To strengthen NIHE rabies laboratory capacity for rabies/*lyssavirus* diagnosis and research

7.3. Specific Objectives: To produce purified N protein of rabies virus for ELISA technique and for production of other biological materials

7.4. Name of Researchers

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– National Institute of Infectious Diseases, Japan.

7.6. Sub-project title: Production of N protein of rabies virus for development of ELISA system and other biological products

7.7. Summary

To date, the rabies surveillance in Vietnam has been mostly based on the clinical survey in both animal and human health. The reason for that are there has been not laboratory system for rabies diagnosis, 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 Sub Department of Animal Health, Ho Chi Minh city). The capacity of those laboratories was limited as well as reagents/kits for rabies diagnosis are sometime not available in the Vietnamese market. Therefore, to step by step set up the rabies laboratory network for better surveillance, investigation and control of rabies in Vietnam, it is necessary to strengthen the national laboratory for rabies/*lyssavirus* diagnosis and research, then extend to the regional, sub regional laboratories. The research was armed to produce purified, recombinant N protein of rabies for ELISA technique and for production of other biological materials.
The construction of N rabies recombinant protein was done by collaboration of NIHE and NIID researchers. We successfully constructed the plasmid inserted N gene of rabies virus which is based on the original sequence of Kyoto strain, and expressed protein on the mammalian cell (293T was used for transfection). The ongoing research for the next year will be cloning the transfected 293 T cell to develop the MSC and WSC for further use; setting up the ELISA system based on the N recombinant protein and evaluation of ELISA

method for screening the antibody against rabies and *lyssaviruses* in serum of vaccinated dogs, human or application of ELISA on sero surveillance of bat *lyssaviruses*.

7.8. Purposes:

Globally each year about 55,000 people die of rabies, most of these deaths occur in Asia and Africa (Knobel, Cleaveland et al. 2005). In recent years, Vietnam as well as the Philippines, Laos, Cambodia, and China have been facing the problem of rapidly increasing human rabies cases. From 2006 to 2011, a total of 560 human rabies cases were reported, among them 90,2% had exposured to dogs and 1,6% had cat bites, interestingly, 8,2% had contacted with dogs or cats during the butchering process, playing with dogs or feeding dogs, and rabies epidemic occurred in 27 provinces in Vietnam. The main transmitters are dogs and cats, no evidence of exposure to other animals were clearly reported. The dog vaccination rate is very low, especially at the remote and rural areas, the transportation, importation/exportation of animals have not been well under control, therefore rabies is easy to transmit from animals to human.

To date, the rabies surveillance in Vietnam has been mostly based on the clinical survey in both animal and human health. The reason for that are there has been not laboratory system for rabies diagnosis, 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 Sub Department of Animal Health, Ho Chi Minh city). The capacity of those laboratories were limited as well as reagents/kits for rabies diagnosis are sometime not available in the Vietnamese market. Therefore, to step by step set up the rabies laboratory net work in Vietnam, it is necessary to strengthen the national laboratory for rabies/*lyssavirus* diagnosis and research, then extend to the regional, sub regional laboratories. The research was armed to produce purified, recombinant N protein of rabies for ELISA technique and for production of other biological materials.

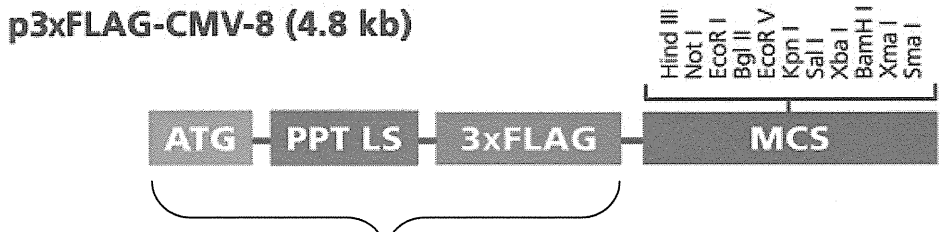
7.9. Methods.

7.9. 1. Design primers for amplification and cloning of N gene into plasmid.

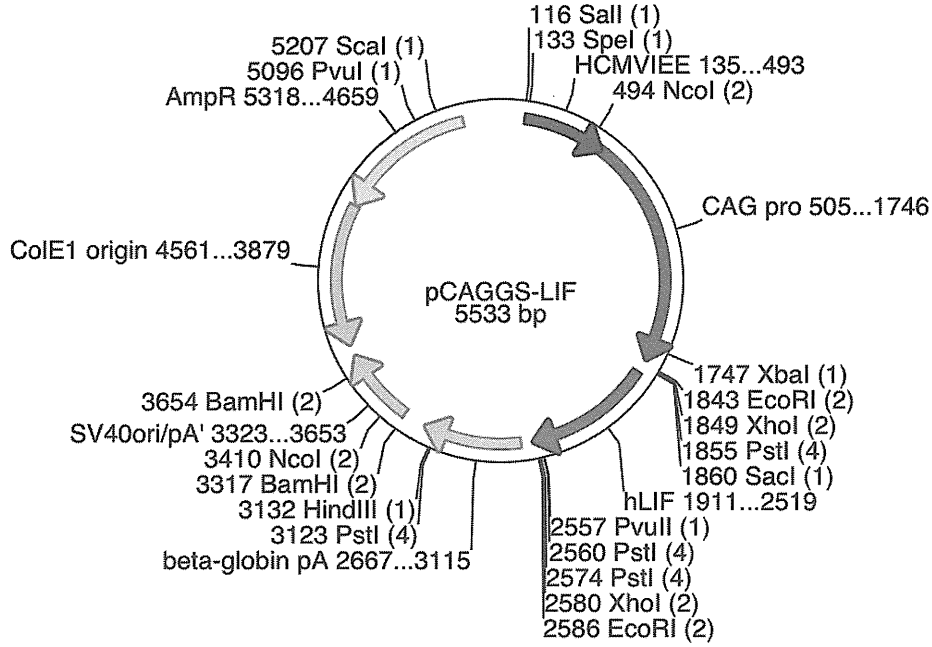
The primers and cloning sites for N gene cloning were designed and determined by GENETX software, version 9 based on the full length sequence of N gene of rabies viruses which were obtained from gene bank.

7.9. 2. Amplification of N gene of rabies virus and insert N gene into plasmid

Amplification of cDNA coded for N protein of rabies virus was done by PCR, using the cDNA of Kyoto strain. The PCR product was then digested with the restricted enzyme and purified by agarose gel separation. The purified, digested DNA of N gene of rabies virus then was inserted into modified pCAGGS vector which was constructed by cutting the PPT – LS – 3XFLAG region of p3xFLAG – CMV8 vector (picture 1), then inserting into pCAGGS vector within the CAG promoter and His region by using the selected cloning sites. The CAG promoter and multi cloning sites of plasmid pCAGGS shows in the picture 2.



Picture 1: FLAG and multi cloning sites of plasmid p3xFlag-CMV-8.



Picture 2: CAG promoter and multi cloning sites of pCAGGS vector.

7.9. 3. ***Transfection:*** The inserted N- modified pCAGGS was transfected into the 293T cell line by fugene transfection reagent as below:

Preparation of cells for transfection.

Adherent cells

One day before the transfection experiment, trypsinize, adjust the cell concentration of 5 x 10^5 cells in a 10 cm culture dish in 12 ml of medium, incubate the cell at 37°C, 5% CO_2 overnight. The cell will achieve the desired density of 50-80% confluence.

Suspension cells

Use freshly passaged cells at a concentration of 5 x 10^6/ ml (12 ml in a 10cm culture dish).

Preparation of fugene reagent and serum, antibiotic free medium mixture.

+ Serum and antibiotic free medium: 600 µl
+ Fugene: 18 µl
+ Vortex in 5 seconds
+ Add 6 µg of plasmid. Vortex 10 seconds then incubate at room temperature for 20 minutes. Add the trasfection mixture onto the cell surface drop by drop without removal of the medium.

Incubation time: 24 – 72 hours at 37°C, 5% CO_2 incubator.

7.9. 4. ***Check the effectiveness of transfection***

After incubation time, the supernatant of transfected 293T cell was collected in a 50ml sterilized tube and clarified by centrifugation at 3,000 rpm/15 minutes then loaded into the FLAG column for purification. Because the N protein of rabies virus was co expressed with the Flag protein. Therefore, it is very convenient to purify N – rabies protein by Flag column. The steps of Flag protein purification as described as below:

Resin preparation

+ Place the chromatography column on a firm support.
+ Rinse the empty column with 0.5 ml of working wash buffer. Allow the buffer to drain from the column and leave residual washing buffer in the column to aid in packing the resin.
+ Thoroughly suspend the resin by gentle inversion. Make sure the ANTI-FLAG M2 affinity gel is a uniform suspension of gel beads. Remove the required amount of resin for use.
+ Immediately transfer the suspension to the column.
+ Allow the gel bed to drain and rinse the pipette used for the resin aliquot with washing buffer. The 50% glycerol buffer will flow slowly and the flow rate will increase during

the equilibration.

+ Add the rinse to the top of the column and allow to drain again. The gel will not crack when excess solution is drained under normal circumstances, but do not let the gel bed run dry.
+ Load three column volumes of Elution Buffer. Let the buffer drain completely. Avoid disrupting the gel bed while loading. Do not leave the column in loading buffer longer than 20 minutes. This step is a mock elution for removal of residual impurities off the column.
+ Wash the resin with five column volumes of washing buffer or until the eluent is at a neutral pH.
+ To equilibrate the resin for use. Do not let the bed run dry. Allow a small amount of buffer to remain on top of the column. Do not allow the resin to remain in washing buffer for extended periods of time (>24 hours) unless an antimicrobial agent (e.g., sodium azide) is added to the buffer.

Column chromatography

+ Load the sample onto the column under gravity flow. Fill the column completely several times, or attach a column reservoir prior to loading for larger volumes. In cases when the FLAG fusion protein is not completely bound (depending on the specific protein and on the loading flow rate), multiple passes over the column will improve the binding efficiency.
+ Usually the sample loading step requires a slow flow to allow binding of the fusion protein to the affinity resin. If the sample volume is up to ~6 ml, it can be loaded in a batch mode by incubation of the resin and sample solution in the column, under a gentle rotation.
+ Wash the column with 10-20 column volumes of washing buffer. This should remove any proteins that are not bound to the M2 antibody. Allow the column to drain completely.

Elution

Elution of FLAG Fusion Proteins by Acid Elution with Glycine - Elute the bound FLAG fusion protein from the column with six 1 ml aliquots of Elution Buffer into vials containing 50-100 ml of 10 Wash Buffer/1 ml of eluent or 15-25 ml of 1 M Tris, pH 8. Do not leave the column in Elution Buffer for longer than 20 minutes. Re-equilibrate column to neutral pH as soon as possible after elution.

7.9. 5. Quality control of recombinant N protein of rabies virus produced.

- *Protein concentration* (using quantitating protein assay kit of BCE – Biorad).
  - Standard BSA is two fold diluted at the concentration of 1,52mg/ml; 0,8mg/ml; 0,4mg/ml and 0,2 mg/ml.
  - Add 100 µl of standard BSA per each concentration to the labeled tubes, concurrently add 100 µl testing protein into previously labeled tube.
  - Add 500 µl of solution A into each above tubes, vortex.
  - Add 4 ml of solution B. Vortex and incubate at RT for 15 minutes. OD of the mixture is determined at 750nm wave length.
  - Determination of protein concentration: Make OD line of standard BSA, protein concentration of the testing protein will be calculated based on the correlation between OD of testing protein and line obtained from OD of standard BSA.
- *The purity of protein: The purity of protein is determined by* SDS-PAGE

First Gel layer (12%):

Gel concentration	10%	12%	15%
H_2O	2,32 ml	**1,92 ml**	1,32 ml
Glycerol 50%	2,0 ml	**2,0 ml**	2,0 ml
Separating buffer 4X pH 8.8	2,0 ml	**2,0 ml**	2,0 ml
Acrylamide 40% (19:1)	2,0 ml	**2,4 ml**	3,0 ml

SDS 10%	80 µl	**80 µl**	80 µl
TEMED	8 µl	**8 µl**	8 µl
Amonium Persulfate (100 mg/ml)	40 µl	**40 µl**	40 µl

The second Gel layer (4.7%)

Ingredients	Volume
H_2O	0.96 ml
Stacking buffer 4X pH 6.8	1.0 ml
Acrylamide 9.5% (19:1) (6.4 g Acrylamide, 3.1 g Bis – Acrylamide)	2.0 ml
SDS 10%	40 µl
TEMED	15 µl
Amonium Persulfate (100 mg/ml)	20 µl

Electrophoresis: 4 hours/ 60V at RT

Gel stain: Coomassie
0.25% Coomassie R-250
50% Methanol
10% acid acetic

Gel distain
5% Methanol
7.5% acid acetic

Replace three times of the distain buffer till the gel becomes white and exposures the standard protein and testing protein bands. Determination of target protein by the molecular weight of protein. The purity of the protein is determined by the appearance of unwanted bands.

- *Determination of protein specificity by ELISA and immune ultra microscopy method*

Using electronic immuno gold lablel assay to determine the specific of antigen produced by recombinant method. The procedure was implemented as below:

- Add 1 drop of Bacitracine 0,01% onto parafine surface, upside down the net coated with Collodion onto the Bacitracine 0,01%. Incubate in 5 minutes.
- Drain briefly the net by absorbent tissue, then add 1 drop of antigen onto the parafine surface. Transfer the drained net to the antigen drop. Incubate in 10 minutes at RT.
- Add 1 drop of anti N – gold onto the surface of parafine paper. Transfer the net to the anti N antobody drop. Incubate in 15 minutes at RT.
- Add 2-3 drops of washing solution - Cacodylate 0,1M onto Prafine surface, transfer net to the Cacodylate 0,1M drop, repeat 3 times.
- Add 1 drop of fixing solution - Glutaraldehyte 0,5%, transfer the net onto the fixing solution, incubate in 5 minutes at RT.
- Drain the net, add 1 drop of staining solution - Uranyl acetate 2%, transfer the net onto the staining solution, incubate in 5 minutes at RT, advoid to exposure with light.
- Drain the net, dry up the net at RT, then observer under electronic microsopy.

7.10. Results

7.10.1. Primers and cloning sites for amplification and cloning of N gene of rabies virus into plasmid

a/ Primers used for cloning PPT – LS region into pCAGGS-P7

- GAATTCaccatgtctgcacttctgat PPT-LS 5'primer (including ECoRI)
- CTCGAGctcgagcttgtcatcgtcatcc PPT-LS 3' primer(including XhoI)

b/ Primers used for cloning N gene of rabies virus into modified pCAGGS-P7

- CTCGAGatggatgccgacaagattgt Rabies-N 5' primer (including XhoI)
- GCGGCCGCGcgaatcactcgaatacgtcttg Rabies-N 3' primer (including NotI)

c/ Cloning sites used for construct the modified pCAGGS-P7 vector

EcoRI and XhoI

d/ Cloning sites used for construct the N rabies- pCAGGS-P7 vector

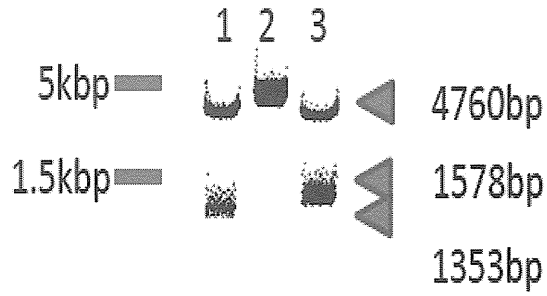
XhoI and NotI

7.10.2. Results of construction of N rabies inserted modified plasmid pCAGGS-P7

Picture 3 shows the sequence of multi cloning sites (MCS), PPP – LS, target gene inserted into vector pCAGGS-P7

Picture 3: MCS sequence and location of N rabies target gene which was inserted into the back bone pCAGGS-P7

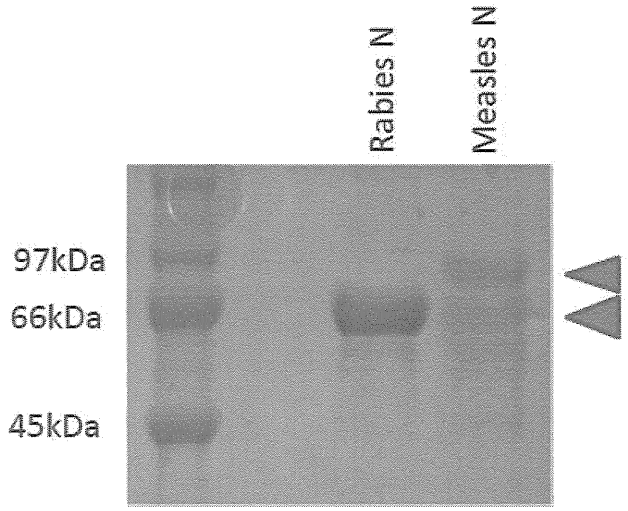
The inserted gene then was checked by Enzyme cutting using by XhoI and NotI, the picture 4 showed the target N rabies gene (lane 1) and Measles N gene (lane 3) were successfully inserted into the pCAGGS-P7. The lane 2 showed the single cut of pCAGGS-P7 inserted Rabies N gene using XhoI.



Picture 4: Electrophoresis of digested N rabies - plasmid pCAGGS-P7 by using restriction enzyme cut at XhoI and NotI.

7.10.3. Results of transfection and production of N recombinant protein

The concentration of N protein was met 1mg/1ml. The purity of the N recombinant protein was showed in picture 5.



7.11. Discussion

The need of development of N rabies protein

Rapid Fluorescence Focus Inhibition Test (RFFIT) is considered as the gold standard to detect and titrate the neutralization antibody against rabies/*lyssaviruses*. But, to perform this

test with large number of samples, it is really a big challenge to the laboratory workers due to time as well as hard working on observing and counting fluorescence focus under x 200 magnification of fluorescence microscope. So, modified RFFIT, an easier and effective method, the Fluorescence Antibody – Virus neutralization (FAVN) test was developed and evaluated by several authors to apply on detection of neutralization antibody in immunized human, animals as well as reservoirs. The absolutely correlative test results of FAVN and RFFIT were demonstrated. Therefore, OIE allowed to use FAVN to determine antibody level against rabies in sero surveillance of dogs or wildlife [10]. Under collaboration of NIHE and NIID, we established the FAVN technique, using different strains of bat *lyssaviruses* to screen and titrate neutralization antibodies against bat *lyssavirus* in order to conduct the research to identify bat *lyssaviruses* in Vietnam as well as to apply on sero surveillance of rabies neutralization antibody in domestic animals for immunization campaign and rabies control and prevention. However, with the big number of serum need to be tested for antibody against rabies/*lyssaviuses* by FAVN, it is still a hard work for laboratory workers. Therefore, if we can develop an ELISA system to screen the presence of anti rabies/*lyssaviruses* in vaccinated human/animals serum or in the reservoirs such as bats, it would be good and widely applied.

Furthermore, recombinant N protein is the media product; we can develop many other biological products from this media agent.

The advantage of using modified pCAGGS vector

The vector encodes three adjacent FLAG epitopes (Asp-Tyr-Lys-Xaa-Xaa-Asp) upstream of the multiple cloning regions. The third epitope includes the enterokinase recognition sequence, allowing cleavage of the 3XFLAG peptide from the purified fusion protein. And this plasmid contains the preprotrypsin leader (PPT) sequence direct secretion of FLAG fusion proteins into the culture medium for purification using anti Flag columns.

The CAG promoter is very strong leader for translation; therefore the high yield of protein will be expressed and released into the supernatant of transfected cells based on the characteristics of PPP – LS FLAG protein.

The future plan

We need to clone the transfected line which can express high yield of N protein and produce the MSC and WSC for further use. Also, the completed procedure for purification of N recombinant rabies protein should be developed as well as checking the quality of the protein through at least three lots of products to ensure that the procedure of production of N recombinant protein is constancy.

N recombinant protein is the prototype product that we can develop many biological products/diagnosis kit based on. Ex: ELISA system to detect anti rabies/*lyssavirus* in serum of vaccinated human, animals and reservoirs such as bats; development of monoclonal antibody against N to produce the Ig – Enzyme or FITC for other diagnosis/ research.

The technique for making recombinant N protein also may be applied for development of recombinant protein of other pathogens.

7.12. Publications: not yet

7.13. **References:**

1. Fugene 6 user manual
2. FLAG purification kit manual
3. K B Hummel, D D Erdman, J Heath and W J Bellini, Baculovirus expression of the nucleoprotein gene of measles virus and utility of the recombinant protein in diagnostic enzyme immunoassays. J. Clin. Microbiol. 1992, 30(11):2874.

7.14. Acknowledgement

We sincerely thank the financial supports from a grant-in-aid of Ministry of Health, Labor and Welfare, the Government of Japan (H23-Shinkou- shitei-020); WHO Vietnam country office; and the Rabies in Asia Foundation, Vietnam chapter.

Research 8

8.1. Project title: Phylogenetic analysis and transmission dynamics of measles and rubella viruses isolated from some outbreaks in the Northern provinces of Viet Nam from 2006 to 2014

8.2. General objectives:

- Identify incidence and trends of measles/rubella
- Describe epidemiological characteristics measles/rubella
- Identify genotypes, subgenotypes and genetic characteristics of measles and rubella viruses circulated in the Northern provinces of Viet Nam from 2006 – 2014.

8.3. Specific Objectives:

- Identify genotypes and genetic characteristics of measles and rubella viruses circulated in the Northern provinces of Viet Nam from 2010 – 2012.

8.4. Name of Researchers

- National Institute of Hygiene and Epidemiology, Viet Nam: Trieu Thi Thanh Van, M.Sc.; Nguyen Phan Le Anh, M.D, M.P.H., Ph.D.
- National Institute of Infectious Diseases, Japan: Dr. Katsuhiro Komase, Department of Virology III

8.5. Affiliation

- National Institute of Hygiene and Epidemiology, Viet Nam.
- National Institute of Infectious Diseases, Japan.

8.6. Sub-project title: Phylogenetic analysis of measles and rubella viruses isolated from some outbreaks in the Northern provinces of Viet Nam from 2010 to 2012.

8.7. Summary: This study will be based on the national epidemiological and laboratory routine surveillance of measles and rubella infection. It is estimated that about 300 throat swabs samples will be collected from patients with rash, fever. Epidemiological, virological, clinical and vaccination information of patients will also be collected by interviewing commune / village health staff, patient's parent, by reviewing hospital records, case investigation forms, outbreak reports and by checking immunization cards and log books. Genetic characterization of wild-type measles virus (MV) was studied using nucleotide sequencing of the C-terminal region of the N protein gene and phylogenetic analysis on 28 throat swab from 4 provinces: Ha Giang, Son La, Ha Tinh and Nghe An in 2010. Genetic characterization of wild-type rubella virus (RV) was studied using 739 nucleotide sequencing (nucleotides 8,731-9,469) of E1 glycoprotein and phylogenetic analysis on 55 isolates from some provinces in the North Viet Nam in 2011. The results showed that 20 strains of MV belonged to genotype H1. The nucleotide sequence homologies of the 20 H1 strains were 98.7%–100%. 17 RV isolates in 2011 were belonged to genotype 2B. The nucleotide sequence homologies of the 17 2B strains were 99%–100%. The report showed that the transmission of genotype H1 of MV and genotype 2B of RV in the North Viet Nam from 2010-2012.

8.8. Purposes: Measles and rubella are similar rash illnesses that may be difficult to differentiate clinically. The routine procedure, laboratory confirmation of suspected cases is based on detection of virus specific immunoglobulin M (IgM) in a single blood after rash onset, molecular techniques such as reverse-transcription polymerase chain reaction (RT-PCR) to dectect viral RNA are often used to complement serologic testing. An important aspect of laboratory surveillance for measles and rubella is the genetic characterization of circulating wild-type viruses to support molecular epidemiologic studies. These studies can help to measure transmission pathways and to clarify epidemiological links during outbreaks. Virologic surveillance that is sufficient to document the interruption of transmission of measles and rubella viruses will be an essential criterion for verification of elimination.

Measles virus (MeV) is a single-stranded, negative-sense RNA virus, belonging to the genus *Morbillivirus*, family *Paramyxoviridae*. Measles is a vaccine-preventable disease, but is still a major killer of infants worldwide. During 2000-2008, global measles mortality

declined by 78%, from an estimated 733,000 deaths in 2000 to 164,000 in 2008. The genome consists of 15,894 nucleotides, which code for the six structural proteins, the nucleoprotein (N), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin (H), and large protein (L), and two nonstructural proteins, C and V. The nucleotide sequences of the L, M, and F genes are much less variable than the sequences of the N, P, and H genes, which have 7%–10% variability. The N and H gene sequences are most commonly used for genetic characterization of wild-type viruses. In particular, one of the most variable parts of the measles genome is the 450-nucleotide region, which codes for the COOH terminal 150 amino acids the N protein, where nucleotide variability can approach 12% between wild-type viruses.

Rubella virus (RV) infection is usually mild or asymptomatic in children and adults. The estimated annual incidence of CRS cases worldwide was 100,000 in 2003. RV is the sole member of the genus *Rubivirus*, in the family *Togaviridae*. The virus has a single-strand, positive sense RNA genome of 9,762 nucleotides (nt) that encodes 2 nonstructural polypeptides (p150 and p90) within its 5_-terminal two-thirds and 3 structural polypeptides (C, E2, and E1) within its 3_-terminal one-third. The E1 glycoprotein is considered immunodominant in the humoral response induced against the structural proteins and contains neutralizing and hemagglutinating determinants. A 739-nt region within the E1 gene (nt 8731 to 9469) is accepted as the minimum amount of sequence information required for molecular epidemiological purposes. Nine rubella virus genotypes (1B, 1C, 1D, 1E, 1F, 1G, 2A, 2B, and 2C) and 4 provisional genotypes (1a, 1 h, 1i, and 1j) based on sequence variation in the 739-nt region have been established.

8.9. Methods:

- ***Specimen Collection:*** Staff members from the Provincial Centre for Preventive Medicine collected throat swab specimens from patients during the outbreaks (from 28 provinces in the North Viet Nam and from 2010 to 2012). Throat swab specimens were obtained according to the WHO procedures for laboratory diagnoses of measles and rubella virus infections and transported to the National Measles Laboratory, National Institute of Hygiene and Epidemiology for processing by standard procedures. To confirm all suspected cases, we used ELISA kits (Siemens, Germany) to detect measles and rubella virus IgM.
- ***Virus Isolation, PCR, and Sequencing:*** Measles virus or rubella virus was isolated by using the Vero/hSLAM cell line. The Vero/hSLAM cell line is now recommended for routine isolation of measles and rubella viruses in the WHO laboratory network. These cells are Vero cells that have been transfected with a plasmid encoding the gene for the human signaling lymphocyte activation molecule (hSLAM) protein. SLAM has been shown to be a receptor for wild type of measles and measles infection of Vero/hSLAM cells results in the characteristic cytopathic effect (CPE). Rubella virus (RV) from clinical samples grow similarly in both Vero and Vero/hSLAM cells but do not cause a reproducible CPE in either. The infected cells were harvested when more than 75% of the culture showed CPE. RNA was extracted from supernatant by using the QIAamp Viral RNA Mini Kit (QIAGEN) according to manufacturer's instructions. Reverse transcription–PCR (RT-PCR) was used to amplify the 634 nt coding for the COOH terminus of the N gene. Meanwhile, RNA was extracted from supernatant without CPE. Reverse transcription–PCR (RT-PCR) was used to amplify either the 185 nt coding for the partial E1 gene to detected rubella virus and then RT-PCR was used to amplify fragment 1 (480 nt), fragment 2 (633 nt) coding for the E1 gene of rubella virus. PCR products were purified by using a QIAquick PCR Purification Kit (QIAGEN). Sequences of the amplicons were obtained by using BigDye terminator version 2.0 chemistry according to the manufacturer's protocol for both sense and antisense strands on an automated 3100 Avant DNA Sequencer (Applied Biosystems). Phylogenetic analyses were performed and trees were generated by using MEGA5 (www.megasoftware.net). The robustness of the groupings was assessed by using bootstrap resampling of 1,000 replicates.
- ***Case investigation:*** Suspected measles / rubella cases (SMR cases) who onset dated 1/1/2010 to 31/12/2012 were investigated with case investigation forms and their throat

swabs samples were taken. Case investigation form includes information of personal information, address, date of rash, contact history, immunization history, symptoms, complications, sample taken date. Study sites were any places in the Northern provinces where outbreaks occurred. From 2010 – 2012, outbreaks happened in 12 provinces including Ha Giang, Son La, Ha Tinh, Nghe An, Vinh Phuc, Ha Noi, Bac Giang, Phu Tho, Bac Ninh, Hung Yen, Thai Binh and Thanh Hoa.

8.10. Results: In 2010, 28 throat swab specimens was collected from patients with rash and fever during the outbreaks in 4 provinces: Ha Giang (3 specimens), Son La (6 specimens), Ha Tinh (1 specimens) and Nghe An (18 specimens). In 2011, 55 thoat swab was collected from patients during the outbreaks in 10 provinces: Vinh Phuc, Ha Noi, Bac Giang, Nghe An, Phu Tho, Bac Ninh, Hung Yen, Thai Binh, Thanh Hoa and Son La.

In 2010, 28 throat swab specimens (Figure 1) were inoculated on Vero/SLAM but only 2 samples have positive result (CPE). The first case was 2 years old child coming from Yen Minh distric, Ha Giang provine. Her throat swab sample collected by our laboratory. The second patient aged 5 coming from Vinh city, Nghe An province. RNA was extracted from supernatant without CPE. RT-PCR was used to amplify either the 185 nt coding for the partial E1 gene to detect rubella virus but result were negative. Then, RNA was extracted directly from 26 clinical samples and RT-PCR was used to amplify the 634 nt coding for the COOH terminus of the N gene and the result shows that:

Table 1: Description of measles viruses in 2010

Patient No.	Patient Age	Address		Result		Strain name
		District	Province	Virus isolation	RT -PCR	
1	2	Yen Minh	Ha Giang	-	-	
2	2	Yen Minh	Ha Giang	+	+	MVi/HaGiang.VNM/16.2010/2
3	1	Yên Minh	Ha Giang	-	-	
4	3	Bac Yen	Son La	-	+	MVs/SonLa.VNM/39.2010/4
5	2	Bac Yen	Son La	-	+	MVs/SonLa.VNM/39.2010/5
6	3	Bac Yen	Son La	-	+	MVs/SonLa.VNM/39.2010/6
7	1	Bac Yen	Son La	-	+	MVs/SonLa.VNM/39.2010/7
8	4	Bac Yen	Son La	-	+	MVs/SonLa.VNM/39.2010/8
9	4	Bac Yen	Son La	-	+	MVs/SonLa.VNM/39.2010/9
10	4	Vinh	Nghe An	-	+	MVs/NgheAn.VNM/50.2010/10
11	5	Vinh	Nghe An	+	+	MVi/ NgheAn.VNM/50.2010/11
12	2	Vinh	Nghe An	-	+	MVs/NgheAn.VNM/50.2010/12
13	4	Vinh	Nghe An	-	+	MVs/NgheAn.VNM/50.2010/13
14	6	Vinh	Nghe An	-	+	MVs/NgheAn.VNM/49.2010/14
15	3	Cua Lo	Nghe An	-	+	MVs/NgheAn.VNM/50.2010/15
16	3	Loc Ha	Ha Tinh	-	+	MVs/NgheAn.VNM/50.2010/16
17	3	Nghi Loc	Nghe An	-	+	MVs/NgheAn.VNM/50.2010/17
18	4	Vinh	Nghe An	-	-	
19	8	Vinh	Nghe An	-	+	
20	8	Vinh	Nghe An	-	+	
21	6	Cua Lo	Nghe An	-	+	
22	5	???	Nghe An	-	+	MVs/NgheAn.VNM/50.2010/22
23	4	Dien Chau	Nghe An	-	+	MVs/NgheAn.VNM/52.2010/23
24	1	Vinh	Nghe An	-	+	MVs/NgheAn.VNM/52.2010/24
25	2	Vinh	Nghe An	-	+	MVs/NgheAn.VNM/51.2010/25
26	1	Vinh	Nghe An	-	+	MVs/NgheAn.VNM/52.2010/26
27	2	Dien Chau	Nghe An	-	+	MVs/NgheAn.VNM/52.2010/27
28	9	Vinh	Nghe An	-	+	

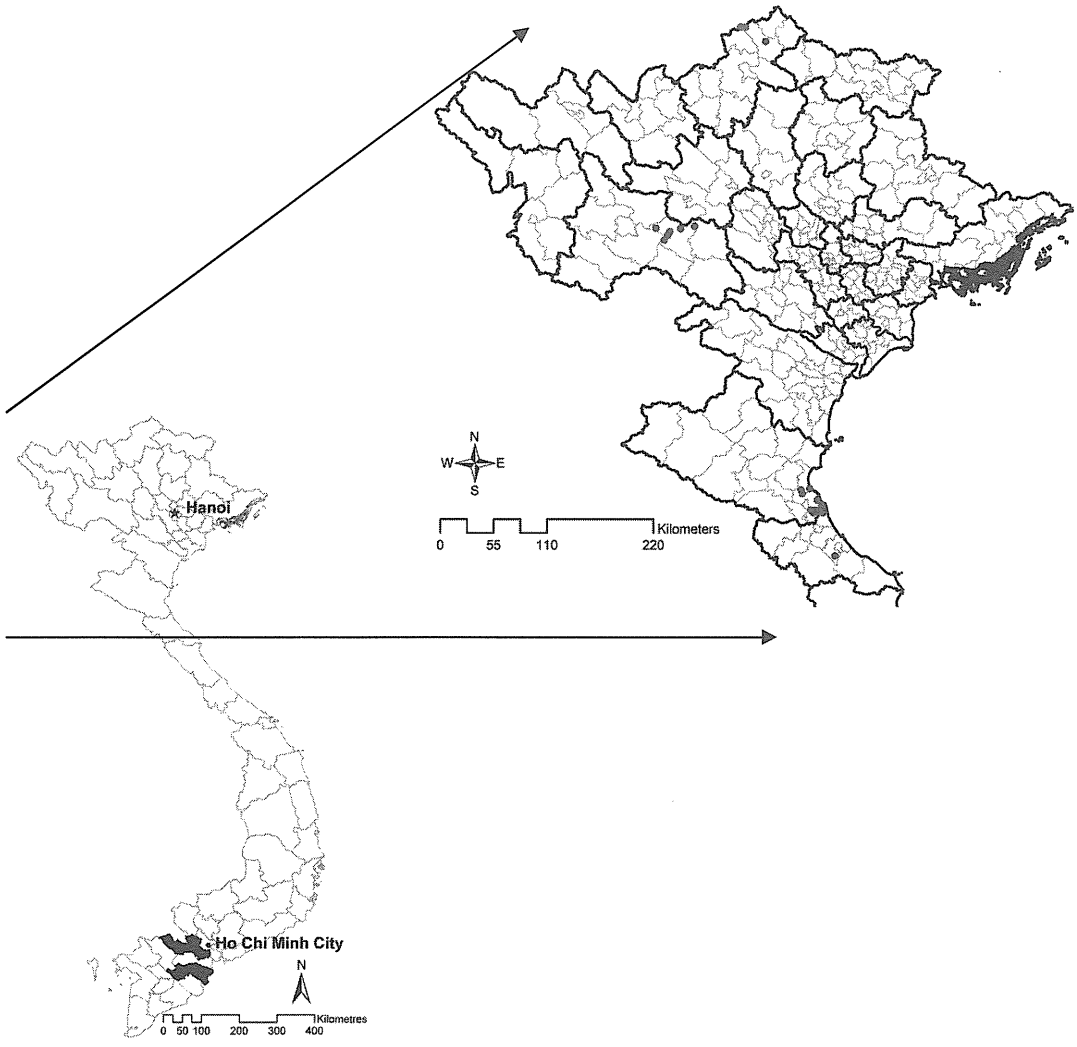


Figure 1: Location of 28 specimens taken in 2010. 25 samples were positive MV (by virus isolation and / or RT-PCR: ● ; 3 samples were negative MV: ●

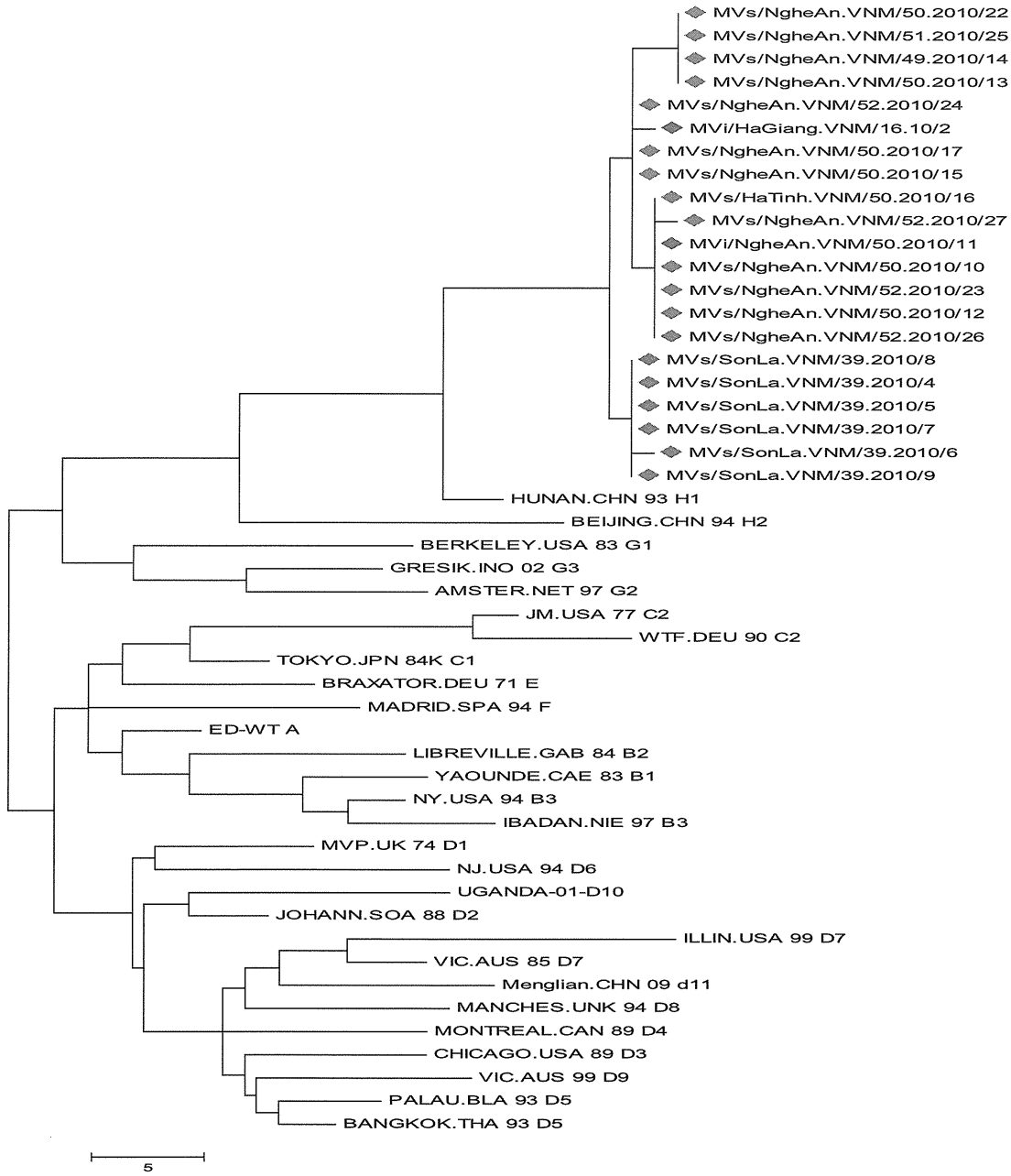


Figure 2. Phylogenetic analysis of the sequences of the nucleoprotein genes (450 nt) of the strains of measles virus in 2010 in the North Viet Nam. The unrooted tree shows sequences from Vietnam viruses compared with World Health Organization (WHO) reference strains for each genotype. MVs, measles virus sequence from throat swab samples. MVi, measles virus sequence from isolates.

The phylogenetic tree is shown that the strains of MV in 2010 in the North Viet Nam grouped with the H1 reference sequences. The nucleotide (nt) difference between the Vietnamese strains in 2010 ranged from 0.0% to 1.3%. The nt difference between the Vietnamese strains in 2010 and H1 reference sequences ranged from 11-13nt (2.4%-2.8%).

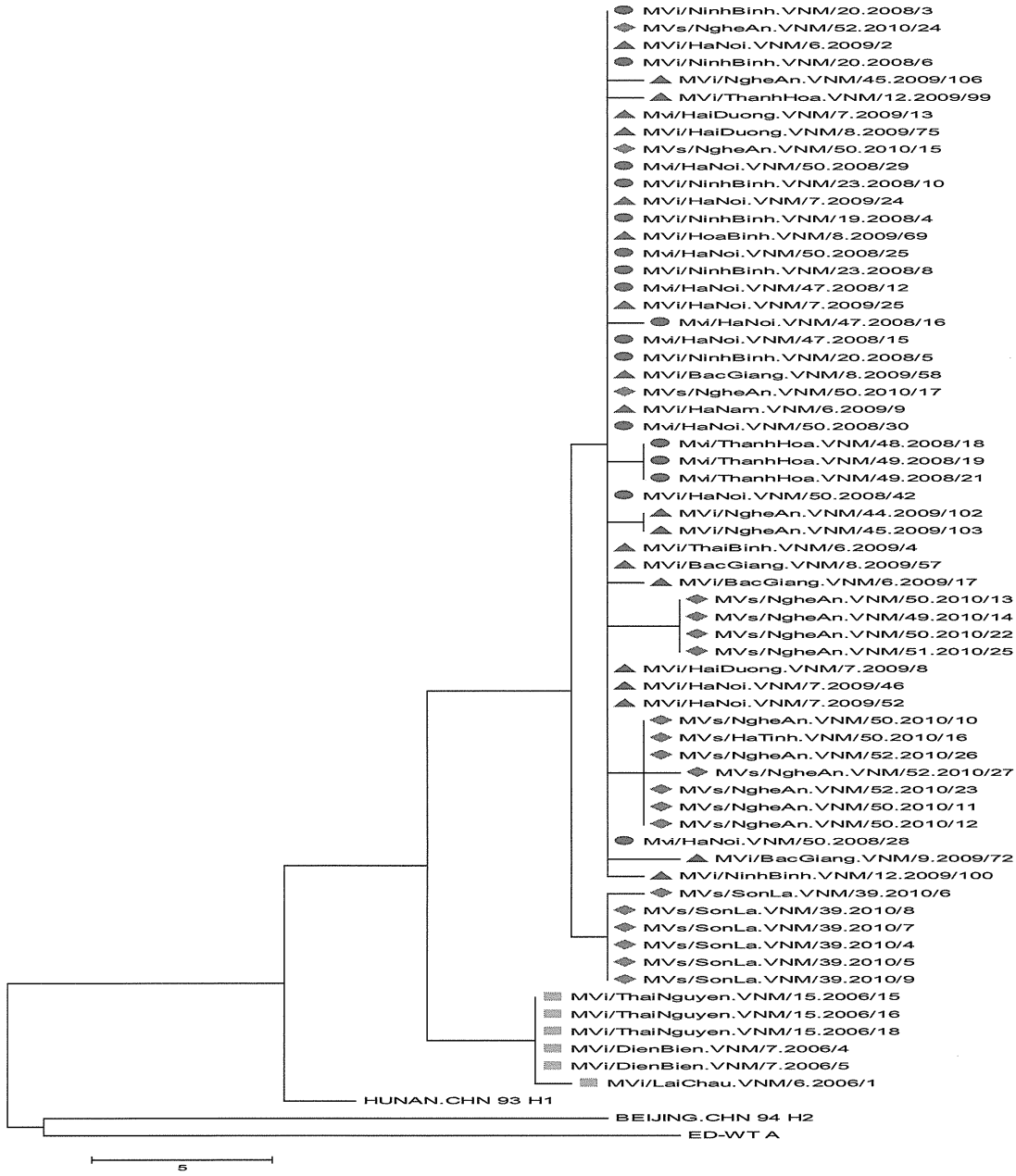


Figure 3. Phylogenetic analysis of the sequences of the nucleoprotein genes (450 nt) of the strains of measles virus from 2006- 2010 in the North Viet Nam and reference strains for H1, H2 and A.
MVs, measles virus sequence from throat swab samples.
MVi, measles virus sequence from isolates.
Sequences from MV isolated in 2006 are indicated by green.
Sequences from MV isolated in 2008 are indicated by blue.
Sequences from MV isolated in 2009 are indicated by red.
Sequences from MV in 2010 are indicated by pink.

It is illustrated in the phylogenetic tree that all of the strains of MV belonged to genotype H1 between 2006-2010. 6 MV isolates in 2006 were belonged to cluster 1; 43 isolates in 2008-2009 and 20 MV in 2010 were cluster 2. The nt difference between the cluster 1 and cluster 2 ranged from 8-10 nt (1.7%-2.2%). The nucleotide sequence homologies of the 69 H1 strains were 97.7%–100%.

55 samples were taken in 2011-2012 and the similar laboratory diagnostic procedures applied but there was no measles positive result.

In 2011, 16 rubella virus isolates were obtained from 55 throat swab samples: 4 from Ha Noi , 2 from Bac Giang, 1 from Thai Binh, 1 from Hung Yen, 1 from Phu Tho, 1 from Bac Ninh and 6 from Vinh Phuc province (Table 2 and Figure 4).

Table 2: Description of rubella viruses in 2011

Patient No.	Patient Age	Address		Result		Strain name
		District	Province	Virus isolation	RT -PCR	
1	3	Yen lac	Vinh Phuc	-	+	Rvi/VinhPhuc.VNM/7.2011/1
2	3	Hoang Mai	Ha Noi	-	+	RVi/HaNoi.VNM/7.2011/2
3	3		Ha Noi	-	+	RVi/HaNoi.VNM/7.2011/3
4	3		Bac Giang	-	+	RVi/BacGiang.VNM/7.2011/4
5			Ha Noi	-	-	
6	2	Nghia Đan	Nghệ An	-	-	
7	1	Nghia Đan	Nghệ An	-	-	
8	3		Nghệ An	-	-	
9	1	Vinh	Nghệ An	-	-	
10	24		Nghệ An	-	+	
11	33		Ha Noi	-	+	
12	20	Tam Nong	Phuc Tho	-	+	RVi/PhuTho.VNM/8.2011/12
13	21	Lap Thach	Vinh Phuc	-	+	RVi/VinhPhuc.VNM/8.2011/13
14		???	???	-	-	
15	3	Tu Liem	Ha Noi	-	-	
16	3	Thanh Xuan	Ha Noi	-	+	RVi/HaNoi.VNM/8.2011/16
17	3	Que Vo	Bac Ninh	-	+	RVi/BacNinh.VNM/9.2011/17
18	3	Van Lam	Hung Yen	-	+	RVi/HungYen.VNM/9.2011/18
19	3	Thanh Xuan	Ha Noi	-	+	
20	3	Cau Giay	Ha Noi	-	-	
21	3	Ba Đinh	Ha Noi	-	+	
22	3	Thanh Tri	Ha Noi	-	+	
23	3	Ba Đinh	Ha Noi	-	+	
24	2	Hoang Mai	Ha Noi	-	+	
25	1		Ha Noi	-	-	
26	3		Ha Noi	-	-	
27			Ha Noi	-	-	
28	2	Hoàng Mai	Ha Noi	-	-	
29	38	Hoàng Mai	Ha Noi	-	+	
30	21	Hoàng Mai	Ha Noi	-	+	
31	25	Hai Bà Trưng	Ha Noi	-	+	
32	3	Hoàng Mai	Ha Noi	-	+	
33	2		Ha Noi	-	-	
34	24		Ha Noi	-	-	
35	2	Hoàng Mai	Ha Noi	-	-	
36	3	Hoàng Mai	Ha Noi	-	+	
37	2	Thai Thuy	Thai Binh	-	+	RVi/ThaiBinh.VNM/11.2011/37
38	14	Thai Thuy	Thai Binh	-	+	

39	29		Ha Noi	-	+	
40	28	Hai Ba Trung	Ha Noi	-	+	RVi/HaNoi.VNM/10.2011/40
41	31	Đong Đa	Ha Noi	-	-	
42	2	Lang Giang	Bac Giang	-	+	RVi/BacGiang.VNM/14.2011/42
43	27	Vinh	Nghe An	-	+	
44	18	Nga Son	Thanh Hoa	-	-	
45	29	Đong Đa	Ha Noi	-	-	
46	24	Vinh Yen	Vinh Phuc	-	-	
47	26	Vinh Yen	Vinh Phuc	-	-	
48	19	Yen Lac	Vinh Phuc	-	+	RVi/VinhPhuc.VNM/13.2011/48
49	25	Tam Duong	Vinh Phuc	-	-	
50	20	Yen Lac	Vinh Phuc	-	+	RVi/VinhPhuc.VNM/15.2011/50
51	21	Vinh Tuong	Vinh Phuc	-	+	RVi/VinhPhuc.VNM/16.2011/51
52	23	Vinh Tuong	Vinh Phuc	-	-	
53	25	Binh Xuyen	Vinh Phuc	-	+	RVi/VinhPhuc.VNM/16.2011/53
54	2	TP Son La	Son La	-	-	
55	1	TP Son La	Son La	-	-	

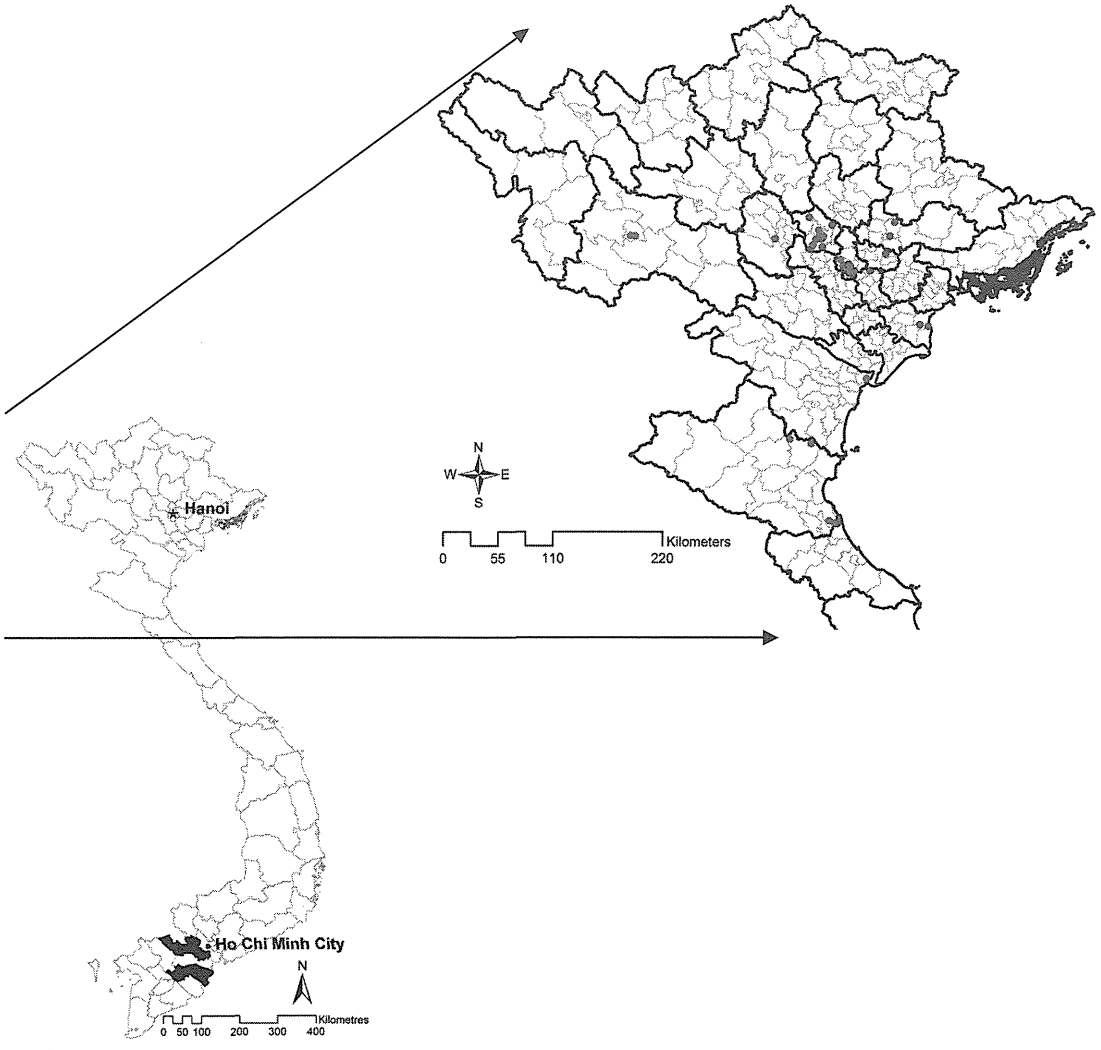


Figure 4: Location of 55 specimens taken in 2011. 31 samples were positive RV (by virus isolation and / or RT-PCR: ●; 24 samples were negative RV: ●

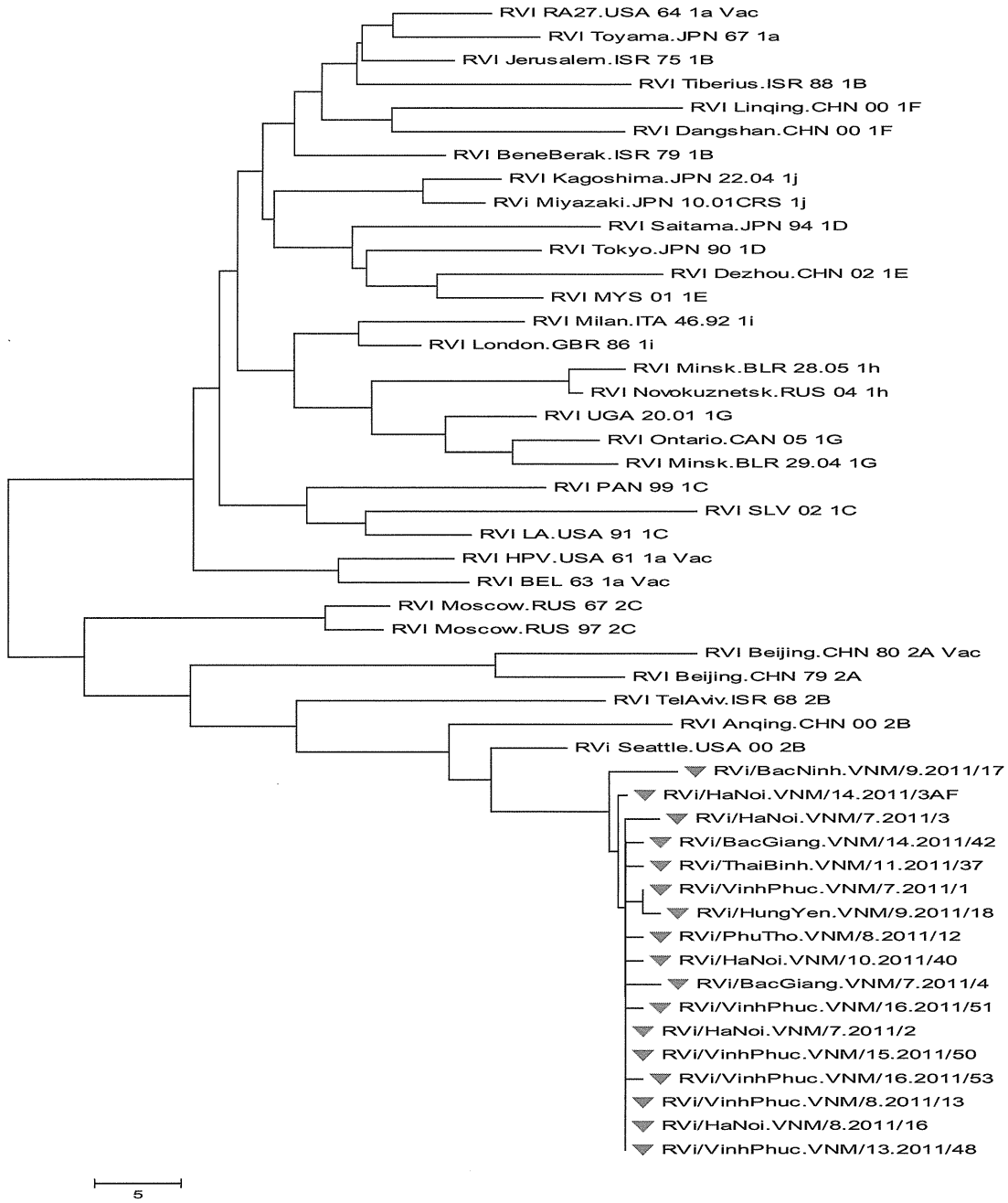


Figure 5. Phylogenetic analysis of the sequences of the E1 gene (739nt) of the strains of rubella virus from 2011. The unrooted tree shows sequences from Viet Nam viruses compared with World Health Organization (WHO) reference strains for each genotype. RVi, rubella virus sequence from isolates.

The phylogenetic tree of RV is shown that the strains of RV in 2011 in the North Viet Nam grouped with the 2B reference sequences. The nt difference between the Vietnamese strains in 2011 and 2B reference (RVI/Anqing.CHN/00_2B) ranged from 3.1% to 3.3%. The nt difference between the Vietnamese strains in 2011 and 2B reference (RVI/TelAviv.ISR/68_2B) ranged from 1.6% to 2.0%. The nt difference between the Vietnamese strains in 2011 and 2B reference (Rvi/Seattle.USA/00_2B) ranged from 4.9% to 5.1%. The nt difference between the Vietnamese strains ranged from 0.8% to 1.4%.

The results from current study have been combined with findings from our previous study to draw the phylogenetic tree of rubella virus in Viet Nam shown in the following figure:

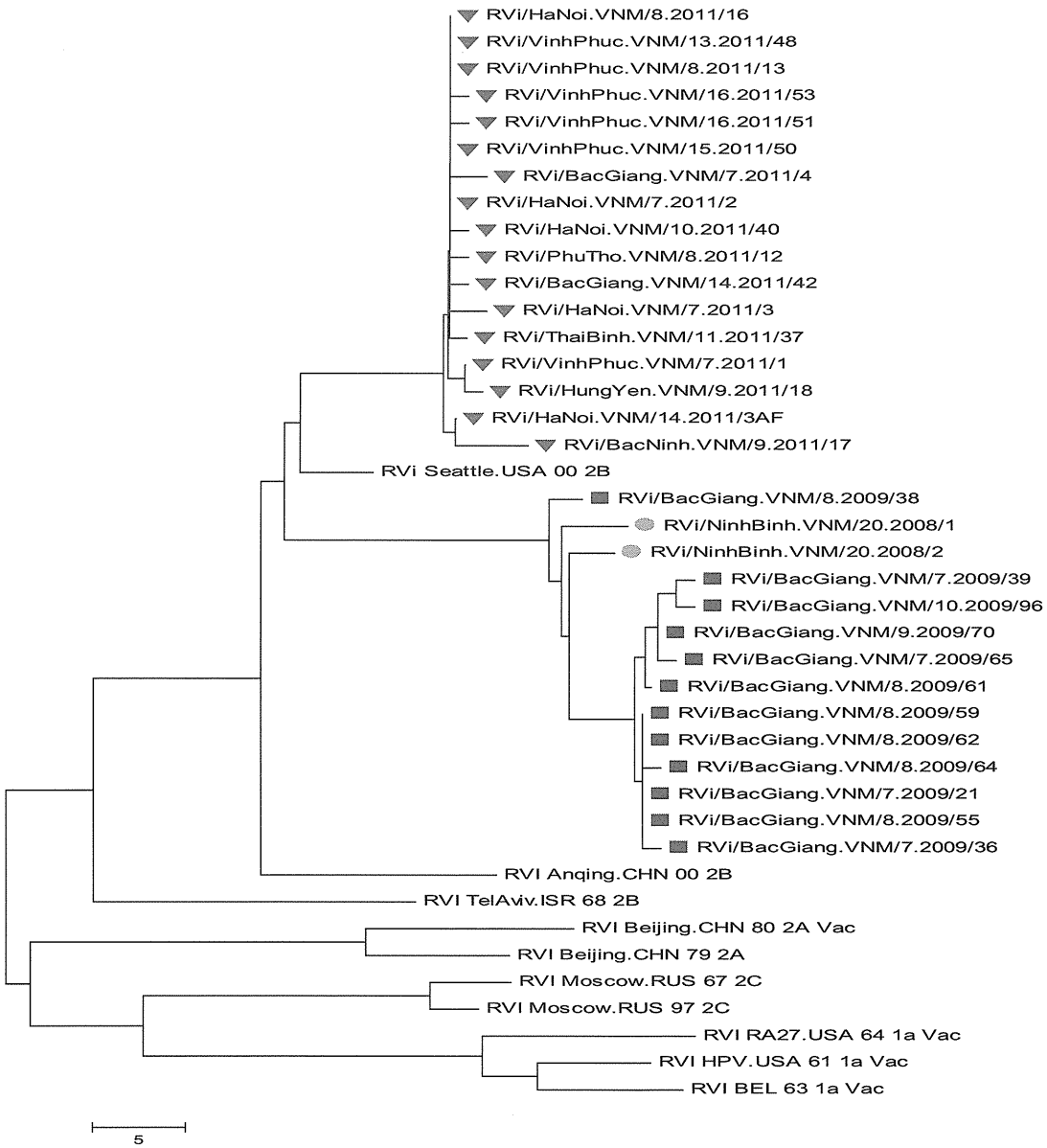


Figure 6. Phylogenetic analysis of the sequences of the E1 gene (739nt) of the strains of rubella virus from 2008-2011 and 3 reference strains for 2B .
Rvi, rubella virus sequence from isolates.
Sequences from RV isolated in 2008 are indicated by green.
Sequences from RV isolated in 2009 are indicated by blue.
Sequences from RV isolated in 2011 are indicated by red.

There was no variation of genotype of RV circulating in the North of Viet Nam in 2008-2011. The phylogenetic tree of RV is shown that all of the strains of RV from 2008-2011 belonged to genotype 2B. The nt difference of these strains of RV was detected but it was minor ranging from 3.4%-4.6%. 14 RV isolates in 2008-2009 were belonged to cluster 1 and 16 isolates in 2011 were cluster 2. The nt difference between the RV strains in 2011 and RV strains in 2008 ranged from 27 – 30 nt (3.7% to 4.1%). The nt difference between the RV strains in 2011 and RV strains in 2009 ranged from 25 – 34 nt (3.4% to 4.6%).

8.10. Discussion: In Vietnam, measles vaccine was introduced into the routine immunization schedule in 1982. National coverage increased during the 1980s, reaching 89% by 1989 and