MINISTRY OF HEALTH
NATIONAL INSTITUTE OF HYGIENE AND EPIDEMIOLOGY
****

THE SECOND PHASE REPORT IN 2012

STRENGTHENING THE RESEARCH CAPACITIES OF THE NATIONAL INSTITUTE OF HYGIENE AND EPIDEMIOLOGY ON SOME NEGLECTED INFECTIOUS DISEASES IN VIETNAM

(Sponsored by
the National Institute of Infectious Diseases, Tokyo, Japan)

Hanoi, March 2013

THE SECOND PHASE REPORT

1. **Project title:** Strengthening the research capacities of the National Institute of Hygiene and Epidemiology on some neglected infectious diseases in Vietnam.

2. **Host agency:**
- Ministry of Health, Vietnam

3. **Implementation agency:**
- National Institute of Hygiene and Epidemiology, Vietnam

4. **Research sponsor**
- National Institute of Infectious Diseases, Japan.

5. **Research titles**

5.1. **Research 1:** Molecular epidemiology, toxin profile and antibiotic resistance of Clostridium difficile infection in some hospitals in the North of Vietnam

5.2. **Research 2:** Clinical epidemiology and molecular characterization of Enterobacteriaceae strains producing Metallo-Beta-Lactamase (including NDM-1) in some hospitals in Hanoi city.

5.3. **Research 3:** Molecular epidemiologic analysis of V. cholerae O1 isolates in Vietnam from 2007 to 2009.

5.4. **Research 4:** The basic and clinical study on Histoplasmosis in Vietnam.

5.5. **Research 5:** Establishment of laboratory diagnosis for leptospirosis and investigation of prevalence of leptospirosis among patients with fever of unknown origin in Northern area of Vietnam.

5.6. **Research 6:** The improvement of the epidemiological surveillance of anthrax in Vietnam.

5.7. **Research 7:** Enhancement of the National Institute of Hygiene and Epidemiology Rabies Laboratory capacity for Rabies/bat Lyssavirus diagnosis and research

5.8. **Research 8:** Phylogenetic analysis and transmission dynamics of Measles and Rubella viruses isolated from some outbreaks in the Northern provinces of Vietnam from 2006 to 2014

5.9. **Research 9:** Epidemiology and molecular characteristics of the hand, foot and mouth disease in the North of Vietnam.

6. **Research duration:**
- 3 year (2012-2014)

7. **Study budget:**
- Total funding for the project is 36.000.000 JPY for 36 months from 2012-2014
- Funding status: National Institute of Hygiene and Epidemiology, Vietnam has received the fund for the second phase which was of 10,000,000 JPY and 494,613.95 JPY that was the remainder of the first phase.

8. **Implementation activities**
- In attached sheets

Hanoi, 15 March, 2013
Signature

Prof. Nguyen Tran Hien, MD., MPH., PhD.
Director
National Institute of Hygiene and Epidemiology, Vietnam.

Research 1

1.1 Project title: First study on *Clostridium difficile* and its infection in Vietnamese hospitals: Prevalence, risk factors and molecular epidemiology.

1.2 Objectives

1.2.1 General objectives: To study *Clostridium difficile* infections in some major hospitals in Hanoi, Vietnam.

1.2.2 Specific Objectives:

- To estimate the proportion of *Clostridium difficile* infections among hospitalized patients presenting with antibiotics-associated diarrhea (AAD) or colitis in some major hospitals in Hanoi, Vietnam.
- To describe risk factors associated with *Clostridium difficile* infections.
- To explore molecular characteristics of *Clostridium difficile* isolated from major hospitals in Hanoi, Northern Vietnam.

1.3 Name of Researchers

- Dr Vu Thi Thu Huong, National Institute of Hygiene and Epidemiology, Vietnam
- Asc. Prof Nguyen Binh Minh, National Institute of Hygiene and Epidemiology, Vietnam
- Ms Tang Thi Nga, National Institute of Hygiene and Epidemiology, Vietnam
- Ms Le Thi Trang, National Institute of Hygiene and Epidemiology, Vietnam
- Dr Tham Chi Dung, National Institute of Hygiene and Epidemiology, Vietnam
- Dr Haru Kato, National Institute of Infectious Diseases, Japan
- Dr Mitsutoshi Senoh, National Institute of Infectious Diseases, Japan
- Dr Keigo Shibayama, National Institute of Infectious Diseases, Japan

1.4 Affiliation

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

1.5 **Sub-project title:** ***Proportion of Clostridium difficile infections in major hospitals in Hanoi, Northern Vietnam***.

1.6 Summary

Antibiotics-associated diarrhea and pseudomembranous colitis are increasingly reported from some hospitals in Ha Noi city, but no laboratory tests for causative agents are available. Thus, this is the first study on the proportion of *Clostridium difficile* infections in Ha Noi, Viet Nam.

We reported the first cases of antibiotic-associated diarrhea and pseudomembranous colitis due to *C. difficile* A- B+ (dominant strains) and *C. difficile* A+ B+ from all 4 hospitals in Hanoi, Vietnam, with other potential causes excluded. This result proved causative role of anaerobic bacteria *C. difficile* in antibiotic-associated diarrhea in Vietnam. In addition, we also provided the first bacteriological evidences on the circulation of 2 bacterial clones, *Clostridium difficile A+ B+* and *Clostridium difficile A- B+* in antibiotics-associated diarrhea in Viet Nam.

1.7 Purposes

Antibiotic-associated diarrhea (AAD) is defined as diarrhea that occurs in association with the administration of antibiotics. The direct toxic effects of antibiotics on the intestine

can alter digestive functions secondary to reduced concentrations of the normal gut bacteria, or cause pathogenic bacteria overgrowth.

The severity of AAD ranges from mild diarrhea to pseudomembranous colitis, megacolon, ileus or even death. AAD imposes a significant financial burden on health care services. Sources of excess costs include prolonged hospital stay, the requirement for isolation and more intensive nursing, treatment costs, laboratory costs and infection control costs. Outbreaks of AAD can occur in both healthcare and community settings, usually caused by *Clostridium difficile*.

Clostridium difficile (C. difficile) is the leading infectious cause of hospital-acquired diarrhea. In Europe and North America, *C. difficile* infections affect more than 60 hospitalized patients per 100,000 (0.06 percent). Recently, *C. difficile* infections have become more frequent, more severe, more difficult to treat, and more likely to recur. In Asia, *C. difficile* has been notified as an emerging and re-emerging pathogen in Japan, China, Singapore and Thailand (Wongwanich, Rugdeekha et al. 2003; Kato, Ito et al. 2007; Huang, Wu et al. 2009; Lim, Ling et al. 2011). These observations have been attributed to a new strain designated BI, NAP1 or ribotype 027. This strain appears to be more virulent than other strains, which may be attributable to increased toxin production compared to conventional strains. Fluoroquinolone use has strongly correlated with the emergence of this strain.

Although it has been lately reported that antibiotics-associated diarrhea syndrome with severe forms increasingly occurs in hospitals in Northern Vietnam (*oral communication with clinicians in Intensive care Units and Emergency Departments of National Hospital of Geriatrics and Bach Mai General hospital*), study on antibiotics-associated diarrhea has never been done in Vietnam yet. In addition, potential contributors of occurring antibiotics-associated diarrhea in Vietnam are the overuse of antibiotics, high levels of antibiotic resistance among bacterial pathogens, inadequate infection control and overcrowding in hospitals.

This project will provide a first estimate of the proportion of the cases of antibiotics-associated diarrhea in Hanoi, Vietnam. The study results possibly highlight that antibiotics-associated diarrhea is an important issue of public health. As a result, it may generate interest of antibiotics-associated diarrhea among health policy makers and clinicians, provide evidence to establish a program for prevention and infection control of antibiotics-associated diarrhea in hospitals in Vietnam.

1.8 Methods.

1.8.1 ***Study subject****:* adults inpatients or outpatients (>=15 years old) with diarrhea or colitis related to recent antibiotic therapy (4 weeks prior to the episodes of diarrhea) were collected consecutively from some hospitals in Hanoi during study period. Those with a diagnosis of acute gastroenteritis, radiation colitis, inflammatory bowel disease, ischemic colitis or diarrhea due to carcinoid tumor, those with a definitive diagnosis of acute diarrhea due to epidemic pathogens such as *Virbio cholera, Salmonella spp, Shigella spp and Staphylococcus aureus;* those with a diagnosis of chronic diarrhea and HIV/AIDS infection were excluded.

1.8.2 ***Study period and sites***: this study was conducted from March 2012 to August 2012 at 3 central hospitals in Hanoi city, Vietnam.

1.8.3 ***Study design****:* Cross-sectional, obseravtional study

1.8.4 Specimen collection:

About 3ml liquid feces were collected in a clean, dry container. The stool specimens were transferred immediately to Laboratory of Anaerobic Bacteria, Department of Bacteriology, NIHE or frozen at -80 oC until transportation..

1.8.5 ***Data collection***: Information on demographic data, recent use of antibiotics, medical history, medical intervention and other potential risk factors were collected by questionnaires.

1.8.6 Laboratory tests to diagnose C. difficile infection:

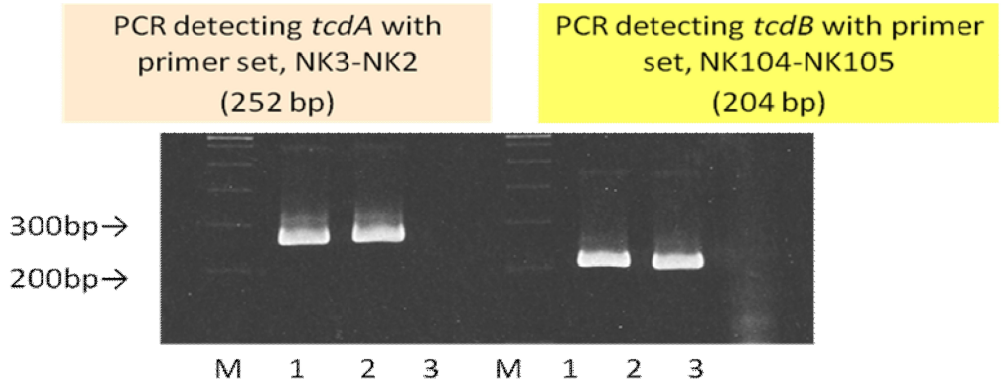
Toxin detection from stool simple by rapid immunoassay test: Toxin A and B of *C. difficile* were detected by comercial kit ImmunoCard Toxins A&B, Nissui, Japan. In comparison with the cytotoxicity assay as the gold standard for the diagnosis of *C. difficile* infection, the sensitivity, specificity, positive predictive values and negative predictive values were 85.22, 93.22, 82.12 and 94.42 for ImmunoCard, which were superior to that of QUIK CHEK (Techlab).

Stool culture for isolation and identification of C. difficile: All stool specimens will be cultured in selective medium containing cycloserine, cefoxitin, and manitol (CCMA medium) for selection of *C. difficile* and cooked meat medium for a back-up and incubate anaerobically for 48h in anaarobic jar or anaerobic bag.

Presumptive identification**:** can be made by demonstrating typical colonies, Gram staining morphology with sub-terminal spores, and characteristic odor.

Definitive identification**:** depends on demonstration by biochemical commercial kits of API 20A along with colony and Gram staining characteristics or positive reaction with *Clostridium difficile* specific antisera using commercial kit CD Check, Nissui, Japan.

Determination of toxigenic C. difficile: C. difficile isolates were cultured in cooked meat medium overnight, then was used directly for toxin detection ImmunoCard Toxins A&B (Nissui, Japan). Simultaneously, bacterial DNA were extracted from pure colonies and used as a template for PCR to detect the presence of the non-repeating sequences of the toxin B gene (tcdB) and the toxin A gene (tcdA) as described previously (Kato, Kato et al. 1999). If toxin A/B test is negative, the tcdB gene will be confirmed by nested PCR on DNA extracted from stool specimens as described previously (Kato, Yokoyama et al. 2005).



Lane M	100 bp ladder
Lane 1	A^+B^+
Lane 2	A^-B^+
Lane 3	A^-B^-

Figure 1. PCR detecting the presence of the non-repeating sequences of the toxin A gene (tcdA) and the toxin B gene (tcdB)

Determination of C. difficile A-B+ and C. difficile A+B+ by amplification of the repeating sequences of the toxin A gene (tcdA):

Multiplex PCR using primers flanking the repeating units of the toxin A gene was done to detect the presence of a deletion of approximately 1.8 kb in the toxin A gene as described previously by Kato and colleagues. This is the portion of the gene that encodes the epitope that reacts with the monoclonal antibody used in the diagnostic EIA kits for detection

of toxin A (PCG-4 epitope). Typical strains (A+B+) do not have this deletion will give a PCR product of 1266 bp, whereas variant strains (A-B+) will produce a PCR product of 714 bp.

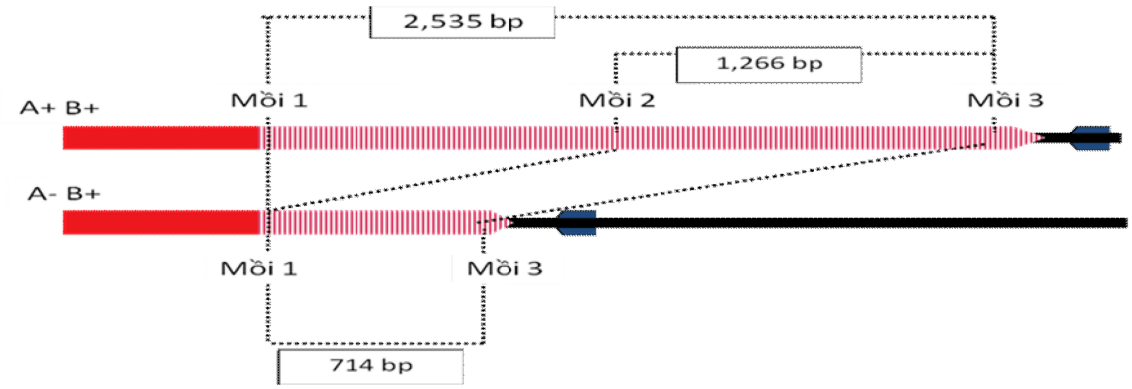


Figure2. Multiplex PCR to differentiate *C. difficile A-B+* isolates from *C. difficile A+B+*isolates.

1.9 Results

Up to date, we have tested 49 stool samples collected from patients with antibiotics associated diarrhea. Ten of 49 samples (20.4%) were positive for *Clostridium difficile* by toxin detection test and/or stool culture.

Descriptive characteristics of patients with *Clostridium difficile* infection were described in the table 1 as below:

Variables	**Freq/mean**	**Percentage**
Gender		
Male	3	37.5
Female	5	62.5
Median age (IQR)	**78**	58-86
Hospital site		
Geriatrics	4	40
Infectious Diseases	4	40
Bach Mai	1	10
Dong Da	1	10
History of hospitalization		
Yes	4	50
No	3	37.5
Unknown	1	12.5
Underlying disease		
Yes	5	62.5
No	3	37.5
Antibiotic use		
Yes	8	100
No	0	0

(Note: Baseline data have not completed yet for 2 samples)

Clostridium difficile infection was detected from all the 4 hospital sites in Hanoi. Positive results were likely associated with the administration of broad spectrum antibiotics (cephalosporin generation 3) and old age.

Toxin detection test and strain genotyping of 10 *Clostridium difficile* isolates showed that 5 *Clostridium difficile* isolates were variant strains A-B+, one isolate was typical strain A+ B+ and 4 strains were nontoxigenic A-B-.

All five variant strains A-B+ were hospital acquired infections whereas one typical strain A+ B+ was isolated from a case of community acquired pseudomembranous colitis. Two patents died possibly due to *Clostridium difficile* infection.

For illustration, we showed here bacteriological evidences on the circulation of 2 bacterial clones, *Clostridium difficile A+ B+* and *Clostridium difficile A- B+* isolated from stool samples in patients with antibiotics-associated diarrhea in Hanoi, Viet Nam (samples coded CDF-0023 and CDF-0104).

- **Toxin detection by rapid immunoassay test**

Toxin detection by commercial kit ImmunoCard Toxins A&B, Nissui, Japan showed that stool samples of CDF-0023 and CDF-0104 were positive for *C. difficile* toxins.

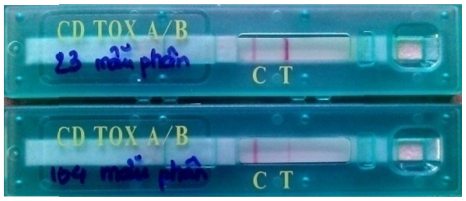


Figure 3. Toxin detection of C. difficile

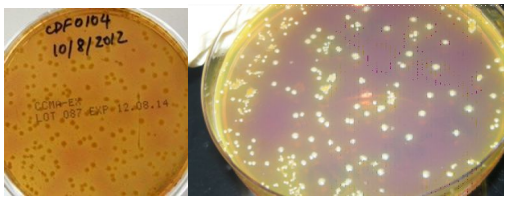


Figure 4. Clostridium difficile colonies on primary CCMA plate

- **Stool culture for anaerobic bacteria C. difficile**

After 48 hours of anaerobic incubation, on CCMA medium, there were irregular, rough yellow colonies with the size of 3-4 mm and horse characteristic odor.

Bacterial morphology showed Gram positive rod, size 1x3 µm, with sub-terminal spores (+++) and free spores (++).

Definitive identification of *C. difficile* was done by commercial kit API 20A (Biomerieux, French) and positive reaction with specific antisera against glutamate dehydrogenase of *C. difficile.*

Singlex PCR detecting the presence of non repeating sequences of toxin A gene (TcdA) and toxin B gene (TcdB) of *C. difficile* showed that sample coded 23 and 104 were positive for TcdA and TcdB

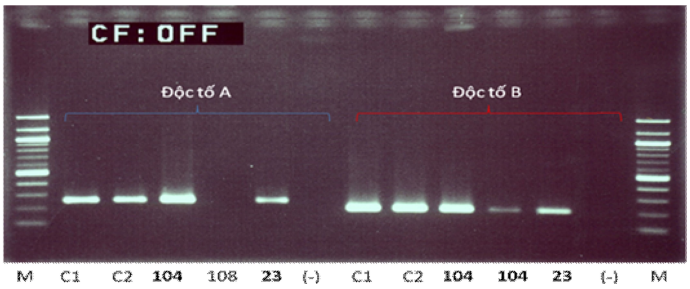


Figure 5. *PCR detecting non-repeating sequences of* toxin A gene (TcdA) and toxin B gene (TcdB) of *C. difficile. (M: standard marker; C1: positive control VPI 10463; C2: positive control G95-01; 104, 108, 23: sample codes; (-): negative control).*

- **Determination of C. difficile A-B+ and C. difficile A+B+ by amplification of the repeating sequences of the toxin A gene (tcdA)**

Multiplex PCR results showed that CDF-0023 isolate was *C. difficile A-B+* while CDF-0104 isolate was *C. difficile* A+ B+.

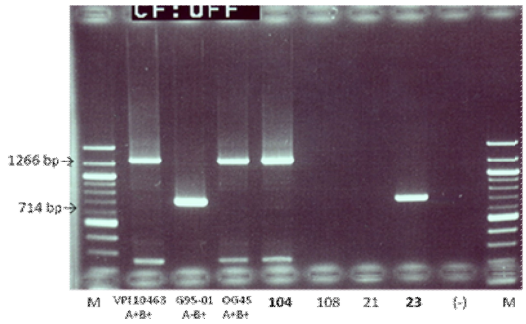


Figure 6. PCR detecting the repeating sequences of the toxin A gene (tcdA). *(M: thang chuẩn; chứng dương VPI 10463 (A+B+); chứng dương G95-01 (A-B+); chứng dương OG45 (A+B+); 104, 108, 21,23: mã số bệnh phẩm; (-): chứng âm)*

1.10 Discussion

C. difficile is an important cause of antibiotic-associated diarrhea and is the causative agent of pseudomembranous colitis. It was previously thought that toxigenic strains of *C. difficile* always produced both toxin A and toxin B, but recent studies have documented the outbreaks of diarrhea associated with toxin A- B+ *C. difficile* at a tertiary care hospital.

In this report, we have reported the first cases of antibiotic-associated diarrhea and pseudomembranous colitis due to *C. difficile* A- B+ (dominant strains) and *C. difficile* A+ B+ from all 4 hospitals in Hanoi, Vietnam, with other potential causes excluded. This result proved causative role of anaerobic bacteria *C. difficile* in antibiotic-associated diarrhea in Vietnam. In addition, we also provided the first bacteriological evidences on the circulation of 2 bacterial clones, *Clostridium difficile A+ B+* and *Clostridium difficile A- B+* in antibiotics-associated diarrhea in Viet Nam.

Current diagnostic methods for *C. difficile* include stool culture, detection of organism-specific glutamate dehydrogenase, detection of toxin B by cell culture or cytotoxicity assay, and detection of toxin A and/or B from stool by immunoassay. Among these methods, stool culture is still the most sensitive method. However, several of the widely used diagnostic tests for *C. difficile* rely solely on the detection of toxin A. From our preliminary results, we suggest that in Vietnam, clinical laboratories should use diagnostic methods that detect both toxin A ad toxin B to avoid false negative results due to variant strains of *C. difficile* A- B+. The relative frequency of toxin A- B+ clinical strains and their relative pathogenicity compared to that of toxin A+ B+ strains warrant further study.

1.11 Publications

1 national paper titled

"Case reports of antibiotics-associated diarrhea and pseudomembranous colitis caused by 2 bacterial clones of *Clostridium difficile A- B+* and *Clostridium difficile A+ B+* in Ha Noi city, Viet Nam"

Published on Journal of Preventive Medicine, Volume 22, Issue 5 (132), page 81-90

1.12 Reference

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1.13 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) and WHO Vietnam country office;

Research 2

2.1 Project title: Molecular characterization Metallo-Beta-Lactamase producing among Gram-negative bacteria strains isolated in the Hospitals of Vietnam

2.2 Objectives:

a) To describe proportion of NDM-1 gene among Gram-negative bacterial isolates resistant to carbapenem
b) To determine the Metallo-Beta-Lactamase production ability among bacteria strains carried NDM-1gene
c) To molecular characterize of NDM-1 bacterial isolates

2.3 Name of Researchers

- Dr. Tran Huy Hoang. National Institute of Hygiene and Epidemiology, Vietnam.
- Dr. Keigo Shibayama. National Institute of Infectious Diseases, Japan.

2.4 Affiliation

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

2.5 Sub-project title: Molecular characterization Metallo-Beta-Lactamase producing among Gram-negative strains isolated in the Hospitals of Vietnam

2.6 Summary:

Background: Infections due to carbapenem resistant Gram-negative bacteria are rising globally, with bacteria containing NDM1 or OXA-48 resistance genes as important examples. In this study, we investigate the proportion of NDM-1 and OXA-48 genes in Multidrug-resistant Gram-negative bacteria isolated from 2010-2012 in three hospitals-Hanoi -Vietnam.

Method: Gram-negative bacteria isolates were studies from three hospitals, (Vietduc, Thanhnhan and Saintpaul) in Hanoi. The presence of carbapenem resistant genes NDM-1 and OXA-48 were established by PCR and sequencing. NDM-1-positive gene strains were typed by pulsed-field gel electrophoresis of XbaI-restricted genomic DNA. Plasmids were analyzed by S1 nuclease digestion. History of travel to abroad of patients had also been investigated.

Finding: From 2010 to 2012, 794 Multidrug-resistant Gram-negative bacteria isolates were collected from three hospitals in Hanoi; we identified 89 (11.2%) isolates with NDM-1 in Vietduc 51, Saintpaul 33 and Thanhnhan hospital 5. NDM-1 was mostly found among *E. coli* (28), *Acinetobacter* (21), *Enterobacer spp* (15) and *Klebsiella* (13). NDM-1-positive bacteria highest were isolated from bronchial fluid (38 isolates) and urine samples (28). Two departments Urology in Vietduc and Pediatric in Saintpaul hospital have highest NDM-1-positive bacteria (28). Male have number infected with NDM-1 higher than female. Patients of less than 9 years and more 60 years tend to be infected with bacterial carrying NDM-1 gene at higher frequency compared to other age groups. All of tested NDM-1 strains were shown to have Metallo-Beta-Lactamase producing. Some *E. coli* isolates in Saintpaul hospital were clonal but most NDM-1 producer in the study was clonally diverse. Preliminary results showing individual or multiple plasmids size in each strain carrying NDM-1 gene suggesting plasmid blaNDM-1 was carried on more than one plasmid. None of patients have any link with country have NDM-1 present. The first time we have also been reported the emergence of OXA-48-Type Carbapenemase-Producing *K. pneumoniae* in Vietnam.

Interpretation: Hospital-acquired infections due to carbapenem resistant strains are a threat to the health care system in Vietnam. Therefore, good surveillance of resistance and proper infection control, as well as monitoring the emergence and spread of the resistant strains, are needed to reduce the impact of resistance and to develop interventions.

2.7 Purposes: To describe molecular characterization of Metallo-beta-lactamase producing bacteria, which are causes of hospital infection in Vietnam in order to put under control and drug-resistant bacteria as a basis for making treatment patients and reasonable effect.

2.8 Methods:

2.8.1 Study design: Cross Sectional Study

2.8.2 Study site:

- Antibiotic Laboratories-Department of Bacteriology, NIHE
- Bacteriology Department II, NIID

2.8.3 Subject: Bacteria strain isolated in hospital-acquired infection from Vietduc, Thanhnhan and Saintpaul hospitals from 2010-2012

2.8.4 Sample size: 794 MDR Gram-negative bacteria strains

2.8.5 Procedure:

- NDM-1 and other carbapenem resistant genes were determined by PCR and sequencing analyze
- Metallo-Beta-Lactamase produce in NDM-1 strains were screened by Japanese and MBL E-test (AB Bio-Merieux)
- The molecular typing were performed done by PFGE using of XbaI restricted genomic DNA
- Plasmid analysis: S1-PFGE, Southern-Blotting and PCR replicon-typing
- Multi locus sequence typing was performed by Bartural Method

2.8.6 Data analyze: NCBI/BLAST and Bio-Numerics software were used to analyze molecular characterization of bacteria strain isolated

2.9 Results

2.9.1 Detection of NDM-1 gen by PCR assay

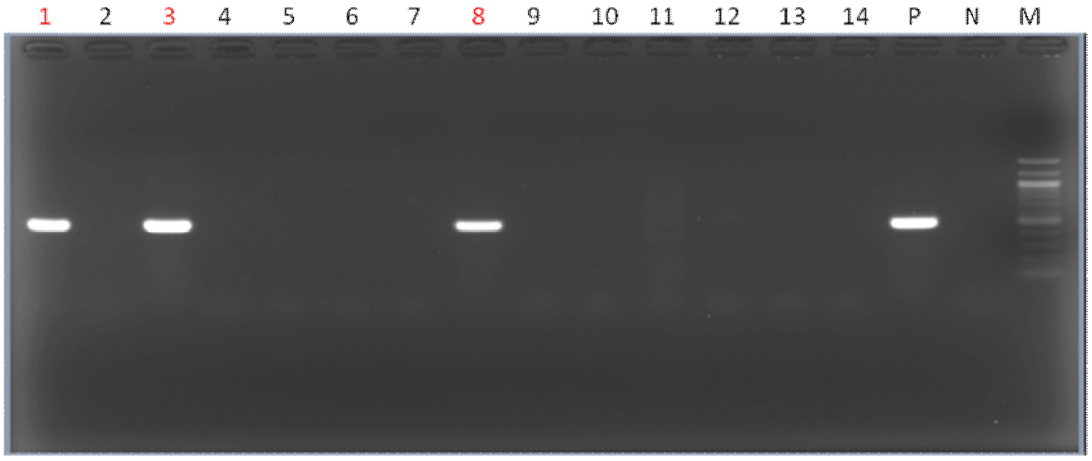


Figure 1: Amplification of NDM-1 gene: 1: *E. coli*; 3: *Proteus spp*; 8: *K. pneumoniae*; P: positive control (DNA extraction from NDM-1 *E. coli* isolated in Japan was kindly provided by Dr. Shibayama in NIID, Japan); N: negative control; M: 100bp ladder.

2.9.2 *Distribution of NDM-1 bacteria strain isolated*

Table 1: Distribution of NDM-1 carrying strains among isolates

Year	*Number of NDM carrying strains/total number of isolates*						
	E. coli	*Klebsiella*	*Citrobacter*	*Enterobacter*	*Acinetobacter*	*Other gram (-)*	Total
2010	7/13	6/36	2/3	7/9	1/37	0/6	**23/104 (22.1%)**
2011	16/24	3/58	3/3	7/10	14/273	1/4	**44/372 (11.8%)**
2012	5/7	4/24	4/6	1/2	6/274	2/5	**22/318 (6.9%)**
Total	**28/44 (63.6%)**	**13/119 (10.9%)**	**9/12 (75%)**	**15/21 (71.4%)**	**21/584 (3.6%)**	**3/15 (20%)**	**89/794 (11.2%)**

From 794 of resistant isolates, 89 (11.2%) of isolated strains were positive with NDM-1 gene. 23 NDM-1 strains were isolated in 2010, 2011 (44) and 22 strains in 2012. Six difference Gram-negative species were carrying NDM-1: Highest was *E. coli* (28), followed by *Acinetobacter* (21), *Enterobacter* (15) *K. pneumoniae* (13), *Citrobacter* (9), and other Gram-negative bacteria were 3 strains.

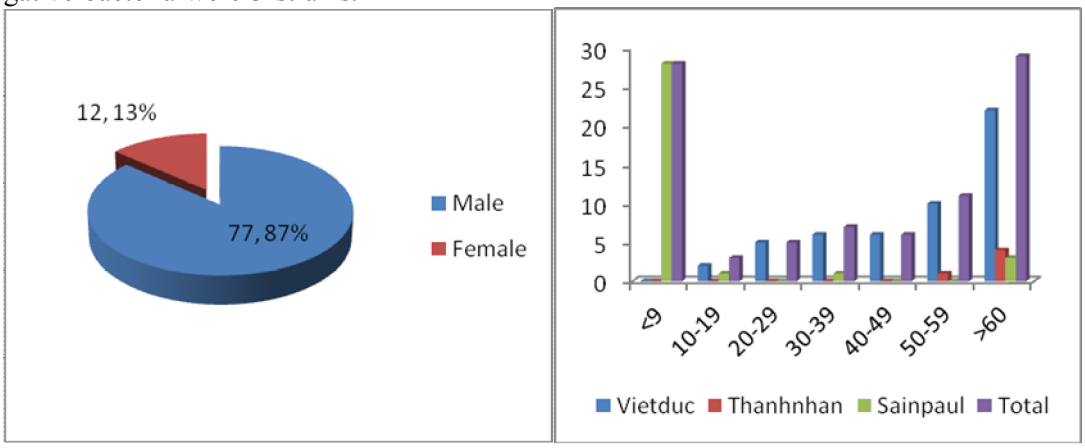


Figure 2: **Distribution of NDM-1 by sex and age groups**

- Higher rate of NDM-1 carrying bacteria was isolated in male patients compared to female
- Patients of less than 9 years and more 60 years tend to be infected with bacterial carrying NDM-1 gene at higher frequency compared to other age groups

Table 2: Distribution of NDM-1 strains by collection sites

Hospital	Urine	Bronchial fluid	Blood	Operation site	Other fluids	Total
Vietduc	27	5	2	8	9	51
Thanhnhan	1	4	0	0	0	5
Saintpaul	0	29	3	0	1	33
Total	**28 (31.5%)**	**38 (42.6%)**	**5 (5.6%)**	**8 (8.9%)**	**10 (11.2%)**	**89 (100%)**

NDM-1-positive bacteria highest were isolated from bronchial fluid (38 isolates), followed by urine samples (28); operation site (8); other fluids (10) and 5 isolates were from blood samples.

Table 3: Distribution of NDM-1 strains by department

Hospital	Urology	Intensive care	Pediatric	Abdominal emergency surgery	Others	Total
Vietduc	28	6	0	4	13	51
Thanhnhan	0	3	0	0	2	5
Saintpaul	0	4	28	0	1	33
Total	**28 (31.5%)**	**13 (14.6%)**	**28 (31.5%)**	**4 (4.5%)**	**16 (17.9%)**	**89 (100%)**

Two departments, Urology in Vietduc and Pediatric in Saintpaul hospital have highest NDM-1-positive bacteria (28), Intensive care unites were 13, other was 16 (general, heart, liver department…) and 4 strains were isolated from department of Abdominal emergency surgery

2.9.3 *Metallo-beta-lactamase producing of NDM-1 bacteria strains*

A: MBL E-test **B: Imipenem- SMA disc**

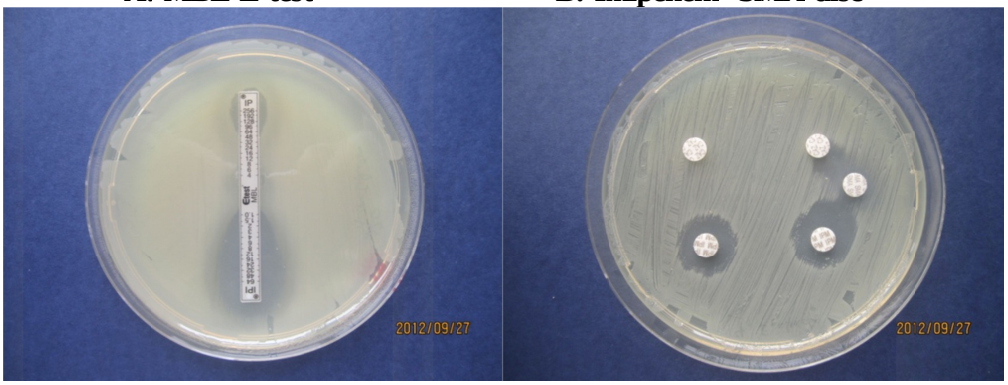


Figure 3: Metallo-beta-lactamase producing among NDM-1 bacteria isolates. **A**. Imipenem-SMA (Japanese kit) contained disc sodium mercapto acetic acid; **B**. MBL Etest (AB Bio-Merieux) contain EDTA

All of selected NDM-1 strains were shown to have Metallo-Beta-Lactamase producing.

2.9.4 *Molecular characterization of NDM-1 bacterial isolates*

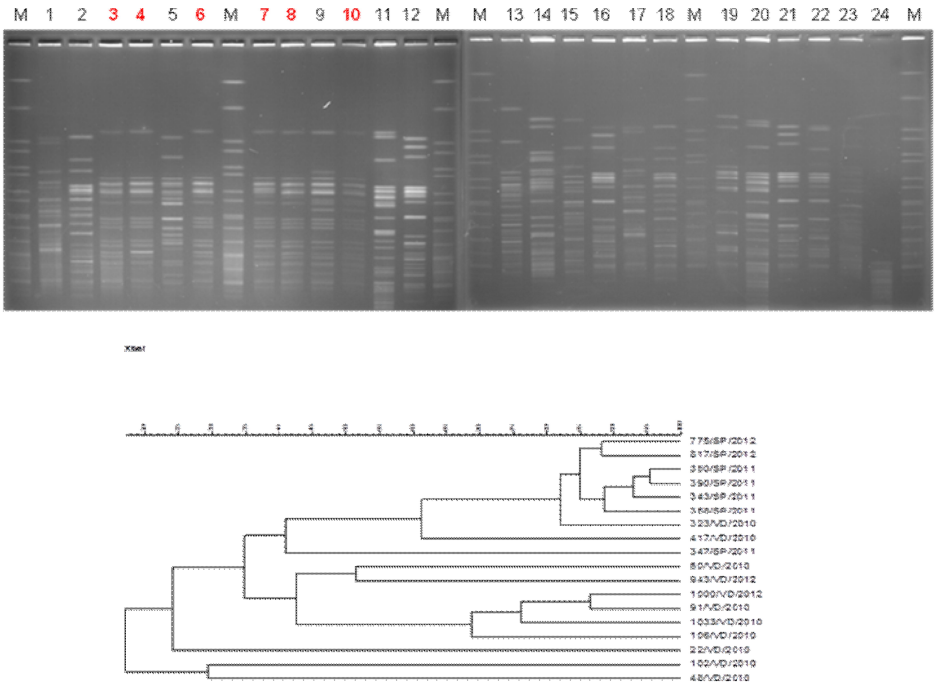
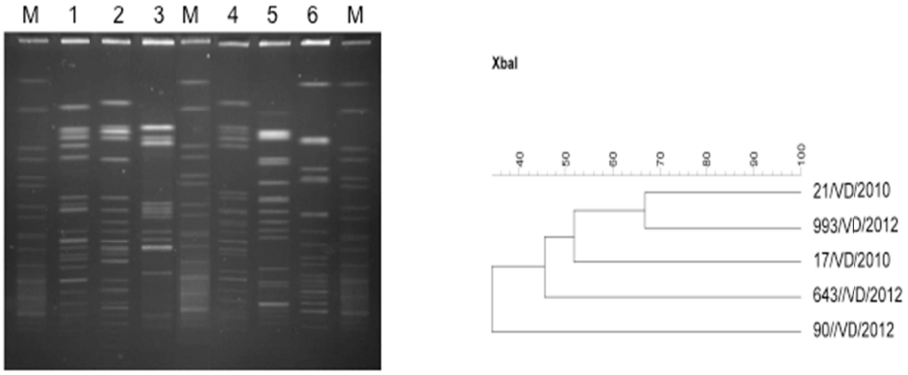


Figure 4: **PFGE profile of NDM-1-*E.coli* strains:** (1: 102/VD/2010; 2: 106/VD/2010; 3: 323/SP/2011; 4: 343/SP/2011; 5: 347/SP/2011; 6: 350/SP/2011; 7: 358/SP/2011; 8: 390/SP/2011; 9: 775/SP/2012; 10:817/SP/2012; 11. 943/VD/2012; 12: 1000/VD/2012; 13:



22/VD/2010; 14: 48/VD/2010; 15: 50/VD/2010; 16: 91/VD/2010; 17: 102/VD/2010; 18: 106/VD/2010; 19: 417/VD/2011; 20: 943/VD/2012; 21: 1000/VD/2012; 22: 1033/VD/2012; 23: 398/SP/2011; 24: 403/SP/2011; M: *Braenderup* H9812).

Figure 5: **PFGE profile of NDM-1-*Klebsiella* strains**: (1: 17/VD/2010; 2: 21/VD/2010; 3: 90/VD/2012; 4: 643/VD/2012; 5: 993/VD/2012; 6: 1037/VD/2012; M: Braenderup H9812

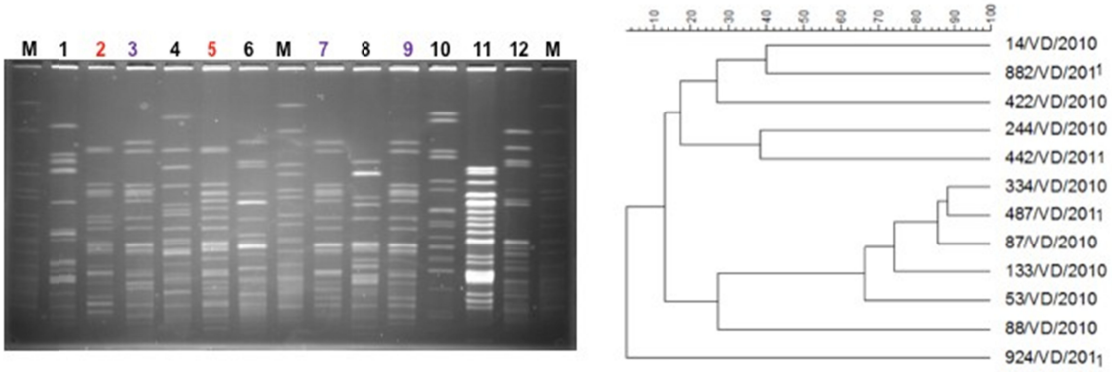


Figure 6: **PFGE profile of NDM-1-*Enterobacter* strains**: (1: 14/VD/2010; 2: 53/VD/2010; 3: 87/VD/2010; 4: 88/VD/2010; 5: 133/VD/2010; 6: 244/VD/2010; 7: 334/VD/2010; 8: 422/VD/2011; 9: 487/VD/2011; 10: 924/VD/2011; 11: 882/VD/Envi/2011; 12: 442/Saint/2011; M: *Braenderup* H9812)

All of the 13 *Klebsiella* and 15 *Enterobacter* had different PFGE profile, with none similar to each other. Most of *E. coli* isolated in three hospitals were difference, however, some NDM-1-positve *E. coli* isolates in Saintpaul hospital from 2010-2012 belonged to a single PFGE profile suggesting clonal spread (Figure 4 to 6).

2.9.5 *Plasmids profile and hybridization results of plasmid carrying NDM-1 gene*

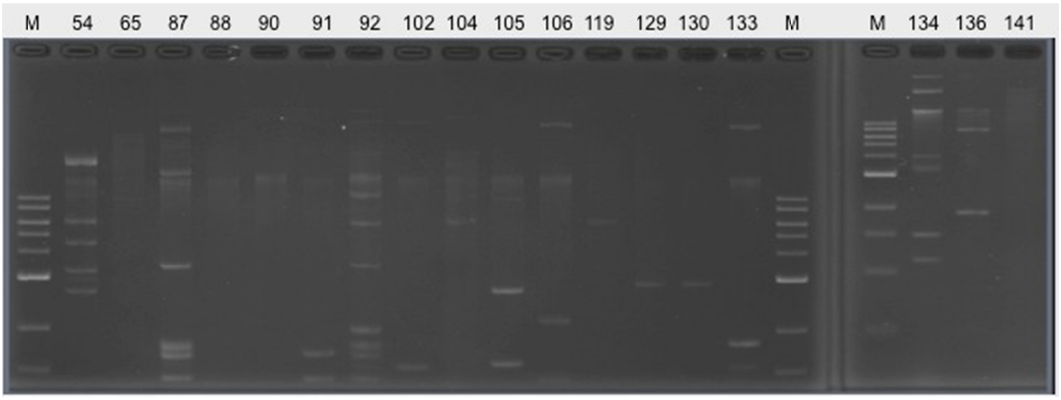


Figure 7: **The difference in plasmid numbers from a selection of NDM-1 isolates.** Many of isolates contained up to seven plasmids.

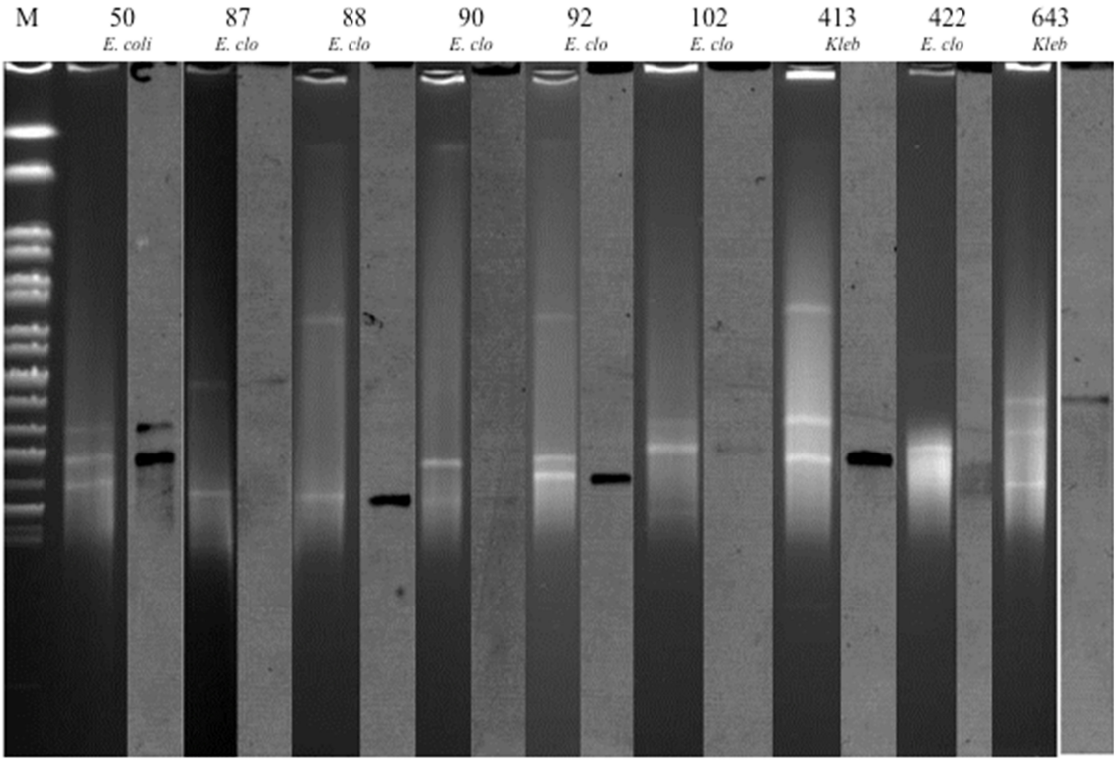


Figure 8: **Preliminary hybridization results of NDM-1 positive strains.** Pulsed-field gel of S1-treated plasmid DNA stained with ethidium bromide, M: *Braenderup* H9812-treated with *XbaI* enzyme and autoradiogram of gel probed with a NDM-1 showing individual or multiple plasmids in each strain carrying NDM-1 gene.

S1 digestion of DNA, and then PFGE and direct probing of the gels with a radiolabelled NDM-1 gene analyzed isolates NDM-1 strains from three hospitals for the location of the blaNDM-1 gene. The isolates typically carried several plasmids, with some isolates having up to eight plasmids (figure 7). Preliminary results showing individual or multiple plasmids size in each strain carrying NDM-1 gene suggesting plasmid blaNDM-1 was carried on more than one plasmid (figure 8).

The emergence of OXA-48-Type Carbapenemase-Producing K. pneumoniae Isolated from Four Hospitalized Patients in Hanoi, Vietnam

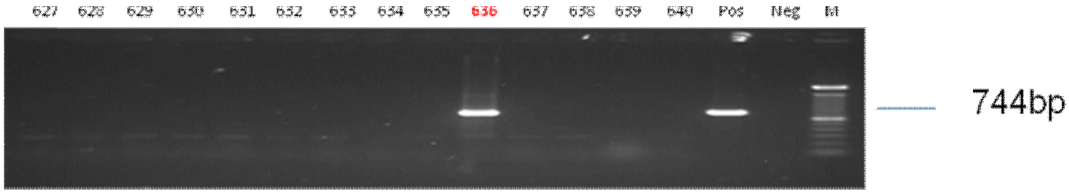


Figure 9: Identification of OXA-48 gene in bacterial isolates. 636: K. pneumoniae; P: positive control (was kindly provided by Dr. Shibayama in NIID, Japan); N: negative; M: 100bp ladder.

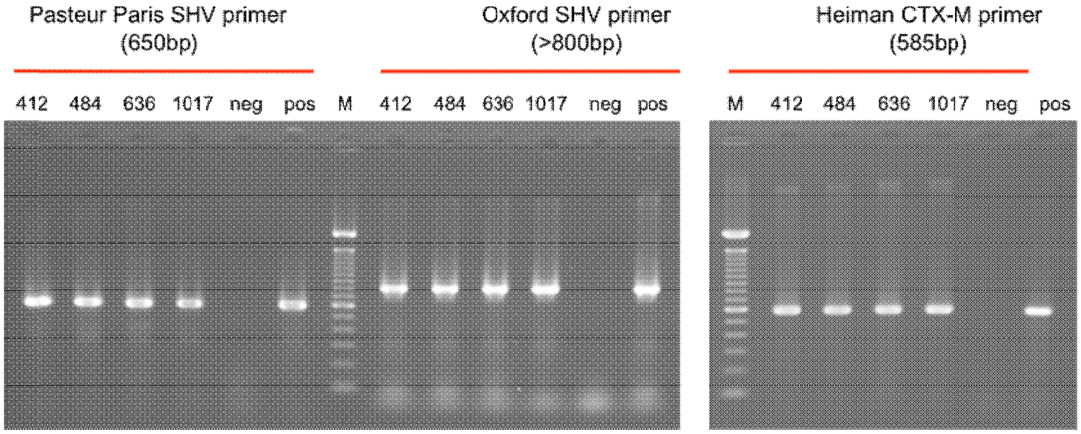


Figure 10: SHV and CTX-M genes of 4 OXA-48-*K. pneumoniae* strains; Positive control: CTX-M (*Shigella* 2009H and 10073); SHV (S. concord 07-670: TEM, SHV)

Four *K. pneumoniae* isolated in Vietduc hospital in Hanoi, Vietnam were positive for OXA-48-type carbapenemase-producing. All of *K. pneumoniae* isolates were resistant to extended-spectrum beta-lactam antibiotics and ciprofloxacin. The MIC for three strains showed resistance to carbapenem at intermediate level. One *K. pneumoniae* strain, isolated from patient No 2, was resistant to carbapenem (table 4). PCR and sequencing analysis showed the presence of OXA-48 genes in all *K. pneumoniae* strains, but NDM1, IMP and VIM genes were absent. In addition, these strains were positive with TEM, SHV and CTX-M genes encoding ESBL (table 4).

Table 4: Key characteristics of patients and their OXA-48 positive strains

Parameter	**Patient 1**	**Patient 2**	**Patient 3**	**Patient 4**
Sex	Male	Male	Male	Female
Age (yr)	48	72	23	24
Admission date	August 2011	October 2011	January 2012	October 2012
Department	ICU	Surgery	ICU	ICU
Hospitalization days	21	20	14	12
Outcome	Alive at discharge	Alive at discharge	Alive at discharge	Alive at discharge
Travel abroad	No	No	No	No
Reason for admission	Traumatic brain injury	Anal fistula	Traumatic brain injury	Traumatic brain injury

Microbiology	Bronchial fluid: *K.pneumoniae*	Abdominal fluid: *K. pneumoniae*	Bronchial fluid: *K. pneumoniae*	Bronchial fluid: *K. pneumoniae*
Beta-Lactamase genes detected	OXA-48, TEM, SHV and CTX-M	OXA-48, TEM, SHV and CTX-M	OXA-48, TEM, SHV and CTX-M	OXA-48, TEM, SHV and CTX-M
Antimicrobial resistance profile	CF,CXM,CAZ,CTX,CFP,CIP,IPM,MEM	CF,CXM,CAZ,CTX,CFP,CIP,IPM,MEM	CF,CXM,CAZ,CTX,CFP,CIP,IPM,MEM	CF,CXM,CAZ,CTX,CFP,CIP,
MIC	Imipenem (2μg/ml), Meropenem (2μg/ml)	Imipenem (8μg/ml), Meropenem (4μg/ml)	Imipenem (2μg/ml), Meropenem (2μg/ml)	Imipenem (2μg/ml), Meropenem (2μg/ml)

2.10 Discussion

Infections due to carbapenem resistant Gram-negative bacteria are rising globally, with bacteria containing NDM1 or OXA-48 resistance genes as important examples. The Gram-negative with NDM-1 and OXA-48 carbapenemase are highly resistant to many antibiotic classes and potentially herald the end of treatment with β-lactams, fluoroquinolones, and aminoglycosides, the main antibiotic classes for the treatment of Gram-negative infections.

PFGE typing did not identify common strain types of *Klebsiella, Enterobater* or in most of *E. coli* strains isolates in three hospitals. However, some of the NDM-1-positive *E. coli* isolates from 2010 to 2012 in Saintpaul hospital were clonal, suggesting that some strains could potentially cause outbreaks. In primary result of southern blotting found several of plasmids size carrying NDM-1 gene, which potential to spread and diversify among bacterial populations in the hospitals. However during the limited time and research funding, we could not molecular characterize of plasmids carrying the NDM-1 gene, therefore these work will be performed in the next phase of this study.

In this study, we were also report the present of OXA-48-type carbapenemase-producing *K. pneumoniae* isolates in Vietnam, these isolates also contained genes encoding for ESBLs. This type of resistance in *Enterobacteriaceae* has been described in Europe, Mediterranean and Africa.

This is the first reports of the high number cases (89; 11.2%) of NDM-1 of Gram-negative bacteria and four OXA-48-type carbapenemase-producing *K. pneumoniae* in Vietnam. None of cases have history of travel to the countries that have present of NDM-1 and OXA-48. The inappropriate use of antibiotics, inadequate infection control and over crowding in the hospitals are important drivers of hospital-acquired infections with MDR bacteria, including NDM-1 and OXA-48in Vietnam.

Hospital-acquired infections due to carbapenem resistant strains are a threat to the health care system in Vietnam, because there are few new anti-Gram-negative antibiotics in the pharmaceutical pipeline and none that are active against these bacteria. Therefore, good surveillance of resistance and proper infection control, as well as monitoring the emergence and spread of the resistant strains, are needed to reduce the impact of resistance and to develop interventions.

2.11 Publications from this research

1. Tran Huy Hoang, Heiman Wertheim, Nguyen Binh Minh, Tran Nhu Duong, Dang Duc Anh, Tran Thi Lan Phuong, Trinh Hong Son, Hidemasa Izumiya, Makoto Ohnishi, Keigo Shibayama, Nguyen Tran Hien (2013). Carbapenem-Resistant *Escherichia coli* and *Klebsiella pneumoniae* Strains Containing New Delhi Metallo-Beta-Lactamase Isolated from Two Patients in Vietnam. *J. Clin. Microbiol*,; 51:1 373-374.

2. Tran Huy Hoang, Nguyen Hoai Thu, Nguyen Binh Minh, Tran Nhu Duong, Tran Van Phuong, Pham Duy Thai, Luong Minh Hoa, Dang Duc Anh, Tran Thi Lan Phuong,Trinh Hong Son , Nguyen Tran Hien (2012). *Citrobacter freundii* carrying New

Delhi-Metallo-Beta-Lactamase (NDM-1) resistant to carbapenem isolated in hospital from 2010-2011. *Journal of Preventive Medicine*. 6(133): 23-30.

3. Tran Huy Hoang, Heiman Wertheim, Keigo Shibayama, Mari Matsui, Nguyen Binh Minh, Tran Nhu Duong, Tran Van Phuong, Nguyen Tran Hien, Trinh Hong Son, Nguyen Tien Quyet and Dang Duc Anh. OXA-48-Type Carbapenemase-Producing *Klebsiella pneumoniae* Isolated from Four Hospitalized Patients in Hanoi, Vietnam. Submitted to *Journal of Global Antimicrobial Resistance* in 1/2013.

2.12 References

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2. **GARP-Vietnam. (2011).** Situation analysis on antibiotic resistance use in Vietnam. *Global Antibiotic Resistance Partnership (GARP)-Vietnam*, Hanoi, Vietnam.
3. **K. K Kumarasamy, M. A Toleman, T. R Walsh, J. Bagaria, F & et al.** Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study**.** *Lancet* 2010. 10: 597-602.
4. **Nordmann P, Cuzon G, Naas T (2009)**. The real threat of *Klebsiella pneumoniae* carbapenemase-producing bacteria. *Lancet Infect Dis*; **9:** 228–36.
5. **Nordmann P, Naas T, and Laurent Poirel L (2011).** Global Spread of Carbapenemase-producing *Enterobacteriaceae. Emer. Infect. Dis*; 17 (10): 1791-1797.
6. **Pitart C, Solé M, Roca I, Fàbrega A, Vila J, Marco F. (2011).** First Outbreak of a Plasmid-Mediated Carbapenem-Hydrolyzing OXA-48 β-Lactamase in Klebsiella pneumoniae in Spain. *Antimicrob Agents Chemother*; 55 (9): 4398–4401.
7. **Poirel P, Héritier C, Tolün V, Nordmann P (2004).** Emergence of Oxacillinase-Mediated Resistance to Imipenem in Klebsiella pneumoniae. *Antimicrob Agents Chemother*; 48 (1): 15–22.
8. **Tran Huy Hoang, Heiman Wertheim, Nguyen Binh Minh, Tran Nhu Duong, Dang Duc Anh, Tran Thi Lan Phuong, Trinh Hong Son, Hidemasa Izumiya, Makoto Ohnishi, Keigo Shibayama, Nguyen Tran Hien (**2013). Carbapenem-Resistant *Escherichia coli* and *Klebsiella pneumoniae* Strains Containing New Delhi Metallo-Beta-Lactamase Isolated from Two Patients in Vietnam. *J. Clin. Microbiol*,; 51:1 373-374.
9. **Yong D, Toleman MA, Giske CG, et al (2009).** Characterization of a new metallo-β-lactamase gene, blaNDM-1, and a novel erythromycin esterase gene carried on a unique genetic structure in *Klebsiella pneumoniae* sequence type 14 from India. *Antimicrob Agents Chemother*; **53:** 5046–54.

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Research 3

3.1. Project title: Molecular epidemiologic analysis of *V. cholerae* O1 isolates in Vietnam from 2007 to 2009

3.2. General objectives

- To analyse molecular epidemiologic of *V. cholerae* O1 strains isolated from cholera outbreaks in Vietnam from 2007 to 2009
-

3.3. Specific objective

- To identify molecular characterization of *V. Cholerae* strains isolated from cholera outbreaks in Vietnam from 2007 to 2009 by using PFGE and MLVA methods
- To investigate relationships between the *V. cholerae* O1 isolates from cholera outbreaks in Vietnam from 2007 to 2009
- To compare molecular characterization of *V. cholerae* O1 isolated from patient and enviroment
- To construct a database and surveillance system of cholera in Vietnam

3.4. Name of researchers:

- Dr. Nguyen Binh Minh (National Institute of Hygiene and Epidemiology, Vietnam).
- Dr. Ngo Tuan Cuong (National Institute of Hygiene and Epidemiology, Vietnam).
- Dr. Nguyen Dong Tu (National Institute of Hygiene and Epidemiology, Vietnam).
- BSc. Nguyen Hoai Thu (National Institute of Hygiene and Epidemiology, Vietnam).
- BSc. Le Thanh Huong (National Institute of Hygiene and Epidemiology, Vietnam).
- Dr. Hidemasa Izumiya (National Institute of Infectious Diseases, Japan).

3.5. Affiliation:

– National Institute of Hygiene and Epidemiology, Vietnam
– National Institute of Infectious Diseases, Japan.

3.6. Sub-project title

Detection and enumeration of Vibrio cholerae in surface water samples in some provinces in North of Vietnam by MPN-mPCR and direct fluorescent antibody methods

3.7. Summary:

MPN test and PCR techniques were combined together to detect the presence of *Vibrio cholerae* and following that, to estimate the bacterial count in a sample. With the MPN-PCR method, a total of 64 water samples collected from enviroment in Haiphong, Thaibinh and Hanoi were examined for the presence of *V. cholerae* . Also we used DFA (direct fluorescent antibody) assay to direct detection of *V. cholerae* in surface water samples. *V. cholerae* O1 and O139 were not detected from all samples by MPN-mPCR and DFA methods. Seventeen of NAG (non-Aglutination) were detected with ToxR positive. The density of NAG in 17 samples ranged from 3.6 up to 1100 MPN/ml. The combined of MPN-PCR method used in proved that is effectiveness for the detection of *V. cholerae* using specific primers as long as its density in the sample compared with identification by conventional plating and biochemical test

3.8. Purpose

- Apply (DFA) assay to detect culturable and nonculturable *V. cholerae* O1 in the aquatic environment of Vietnam.
- Development of quantitative MPN-mPCR (most probable number - multiplex PCR) method to enumerate *Vibrio choleare* in water environment

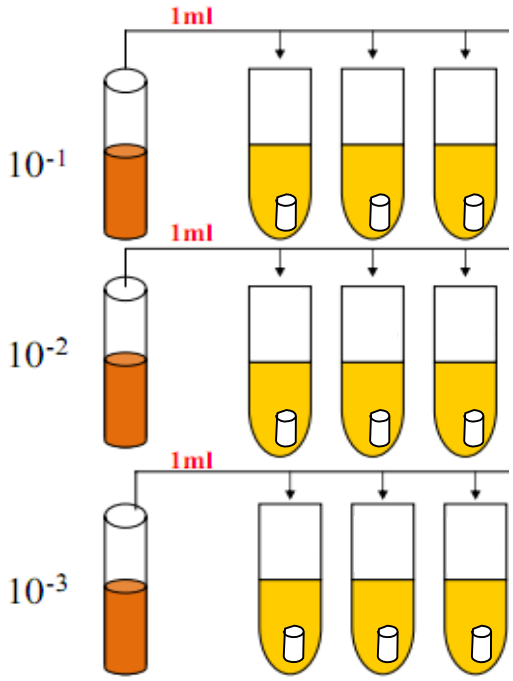
3.9. Methods

3.9.1. Most Probable Number (MPN) method

Most Probable Number (MPN) test applies the use of statistical mathematics to obtain quantitative data on concentration of bacteria in a sample. MPN test and PCR techniques were combined together to detect the presence of *Vibrio cholerae* and following that, to estimate the bacterial count in a sample.

The water sample to be tested is prepared in 10-fold dilution series, and then 1ml samples of each dilution are inoculated into triplicate alkaline peptone broth culture tubes for incubation. A typical design uses three replicates with a three-$\log_{10}$ unit dilution series. Following incubation, all tubes are examined for turbidity and the pattern of growth in the tubes is scored against a table of such values (see Annex). If all tubes showed growth, then the results will be notes as 333. If only one tube in each replicate shows growth it would be denoted as 111. The pattern of growth is then read from the table to provide the most probable number and 95% confidence interval. By this result of 210 would reflect an MPN of 21.

Detail steps: Centrifuge 100 ml of surface water sample at 11,200xg for 10 min. Following that, 90ml supernatant from each tube were discarded. The pellets were resuspended with a vortex mixer. The remaining water sample is serally 10x diluted in salin or PBS. 10ml x3 of diluent are also inoculated in 3 tubes of APW. The final dilution would be 1/100, 1/1000, 1/10000. A total of 9 tubes of APW are need for one sample. Template DNA is prepared from the culture, and then subjected to PCR.



3.9.2. Multiplex-PCR

The specific gens for *V. cholerae* O1: V.O1, V.O139, ctx A, ToxA were detected by multiplex-PCR. PCR was carried out in a 0.2 ml microcentrifuge tubes with 24 「「l of the PCR mixture containing 10 「「l each of forward and reverse primers (20 「「l and 1 「「l (ca. 0.1 「「g) of template DNA by using Go-Taq® Green Master Mix (Promega, Madison, Wis.). The solution was mixed, centrifuged briefly, and placed in an automated Eppendorf PCR Thermal Cycler (Hamburg, Germany). PCR amplification conditions were as follows: initial denaturation at 94°C for 2 min, and 30 cycles of 1min-denaturation at 94°C, 1min-annealing at 60°C, and 1 min-extension at 72°C with a final extension step at 72°C for 7 min at the end

of 30 cycles, followed by maintenance at -4°C. PCR products were separated by 2% agarose gel electrophoresis in 1xTAE buffer (40 mM Tris-acetate, 1mM EDTA, pH 8.0), The molecular masses of the amplicons were determined by comparison with molecular mass markers of Ladder 100bp. Amplification products were stained with ethidium bromide, and visualized under UV light. The primers used in this study are shown in Table 1.

Table 1. Primers used in the multiplex PCR

Primers	Sequences(5` to 3`)	**Target gene**	**Amplicon size (bp)**
VCO1 F2-1 VCO1R2-2	5'GTT TCA CTG AAC AGA TGG G 3' 5'GGT CAT CTG TAA GAT CAA C 3'	O1	192
VCO139F2 VCO139 R2	5' AGC CTC TTT ATT ACG GGT GG 3' 5'GTC AAA CCC GAT CGT AAA GG 3'	O139	449
AX2 AX3	5' CGG GCA GAT TCT AGA CCT CCT G 3' 5' CGA TGA TCT TGG AGC ATT CCC AC 3'	*ctxA*	564
101F 837R	5'CCT TCG ATC CCC TAA GCA ATA C 3' 5'AGG GTT AGC AAC GAT GCG TAA G 3'	*ToxR*	779

3.9.3. Direct Fluorescent assay (DFA)

Detection of *V. cholerae* by fluorescent antibody staining has been shown to be highly sensitive in detecting both culturable and non-culturable organisms.

The cholera DFA Test consists of a monoclonal antibody, specific for the A antigen of O1 lipopolysaccharide in the outer membrane of *V. cholerae* O1 that is directly labeled with fluorescein isothio cyanate (FITC) for the rapid, simple detection and enumeration of *V. cholerae* O1 in water samples.

Principle of assay

The test kit is comprised of the cholera DFA reagent and two control reagents. Water samples are concentrated and a sample is fixed onto a microscope slide. The test sample and control sample are then incubated with the DFA reagent. If the sample contains *V. cholerae* O1, the FTTC-labeled monoclonal antibody will bind *V. cholerae* O1. After washing, the slide is examined under the fluorescent microscope.

Sample preparation

- Water sample of 100 to 500 ml collected in a clean container. If the water sample is turbid, it should be filtered through a 10.0 µm filter. Temperature, salinity were measured with a meter Instrument.
- Using negative pressure from pump or other vacuum source, concentrate the 100 to 500 ml water sample. Place the filter on a clen petri dish and add 1 ml of sterile PBS onto it in order to obtain a thick suspension of organisms.

Procedure

- Prepare Cholera O1 DFA reagent and sample. All materials should be at room temperature.
- Make thin smear of resuspended sample by adding 5 µl on a well, then spreading the contents to cover the well.
- Make a thin smear of the positive control by adding a small drop of the control on a well,
- Then spreading the drop to cover the well. Make a similar thin smear of the negative control. Make a similar thin smear of the sample by adding approximately 5 µl of the sample to the slide and spreading.
- Air dry or incubate at 37^0C until dry.
- Add 5 µl of absolute ethanol or methanol to each control or sample well to fix the smear, then air dry.
- Add 10 µl of reconstituted Cholera O1 DFA reagent to each well.

- Place slides in a covered, moist chamber, and incubate at 35^0C for 30±5 minutes. Protect from light.
- Rinse the slides thoroughly with PBS. Protect from light.
- Absorb off excess moisture using a blotter paper.
- Add a drop of Fluorescent Mounting Medium on the slide and cover with a 22x50mm, No. 1 coverslip.
- For the best results, the slides should be read immediately at a magnification of 1000 X with oil immersion. Equipvalent readings may be obtained if the slides are read within 24 hours. The slides must, however, be kept cool, in the dark, and sealed, or kept humid to prevent drying.

3.10. Results

3.10.1. MPN-mPCR analysis

Multiplex PCR were used to identify the specific genes of *V. cholerae* : V.O1, V.O139, *ctx A, ToxR*. The data showed that the 44 of water samples of tested were all negative for V.O1, *ctx A* and 17 of them positive for *ToxR* genes.

Table 2: Detect the specific genes of *V. cholerae by* Multiplex PCR

Locals	Number of samples	Presence of genes			
		ctxA	V.O1	V.O139	*ToxR*
Haiphong	28	-	-	-	5 pos
Hanoi	30	-	-	-	12 pos
Thaibinh	06	-	-	-	0 pos
	Total: 64	-	-	-	17 pos

Table 3: Density (MPN/ml) of NAG in water samples by MPN-mPCR (ToxR positive)

	No	Locals	Results of MPN/ml			
			September 2012	**October 2012**	**November 2012**	**December 2012**
Hai Phong (28 samples)	1	Hồ Tam Bạc	0	0	0	0
	2	Hồ Sen	0	0	3.6/*ToxR* (+)	0
	3	Hồ An Biên	0	93/*ToxR* (+)	0	0
	4	Mương Hòa Nghĩa		23/*ToxR* (+)	0	0
	5	Mương Hợp Đức		3.6/*ToxR* (+)	0	0
	6	Mương bãi rác			0	0
	7	Đầm Phả Lễ 1			0	0
	8	Đầm Phả Lễ 2			0	0
	9	Mương Lập Lễ 1			6.2/*ToxR* (+)	0
	10	Mương Lập Lễ 2			0	0
Ha noi (30 samples)	11	Sông Kim Ngưu Cầu Lạc Trung		0	23/*ToxR* (+)	0
	12	Sông Kim Ngưu Tam Trinh		0	0	9.2/*ToxR* (+)
	13	Hồ Thanh Nhàn		0	0	0
	14	Hồ Yên Sở Hoàng Mai		75/*ToxR* (+)	1100/*ToxR* (+)	3.6/*ToxR* (+)
	15	Sông Tô Lịch ngã Tư sở		0	43/*ToxR* (+)	0
	16	Hồ Linh Đàm		20/*ToxR* (+)	0	0
	17	Sông Tô Lịch Đại Kim		0	0	3.6/*ToxR* (+)
	18	Cầu Bươu Hà Đông		0	0	35/*ToxR* (+)
	19	Cầu Sông Nhuệ		43/*ToxR* (+)	0	1100/*ToxR* (+)
	20	Ao rau muống		0	0	9.2/*ToxR* (+)

Thai Binh (6 samples)	21	Sông bệnh viện huyện Tiền Hải		0		
	22	Sông gần chỗ cất vớ Tây Tiến		0		
	23	Sông gần PTTH Nam Tiền Hải		0		
	24	Sông 4 cống Nam Hưng		0		
	25	Sông bãi rác Nam Hưng		0		
	26	Sông cống ông tiêu		0		
Total samples: 64			**3**	**21**	**20**	**20**

MPN test and PCR techniques were combined together to detect the presence of *Vibrio cholerae* and following that, to estimate the bacterial count in a sample.The density of NAG in all samples ranged from 3.6 up to 1100 MPN/ml.

Table 4: Number of NAG with ToxR positive detected by MPN-mPCR

No	Locals	September	October	November	December
1	Haiphong	3 Negative	3 Positive/5	2 Positive/10	10 Negative
2	Hanoi		3 Positive/10	3 Positive/10	6 Positive/10
3	Thaibinh		6 Negative		
	Total	**3 (-)**	**6 Positive/21**	**5 Positive/20**	**6 Positive/20**

3.10.2. Result of direct fluorescent antibody (DFA) assay:

Vibrio cholerae O1 and O139 were not detected from all 64 samples by direct fluorescent antibody (DFA) assay.

3.11. Discussion

mPCR assay enables the detection of *V. cholerae* in sutface water samples using their specific primers. In general, cholera toxin gene confers the virulence factor of *V. cholerae*, giving this bacterium the ability to cause disease in human. This gen was normally found in *V. cholerae* of the O1 and O139 serogroups. The purpose of detecting *ctxA* during MPN-PCR was to analyze whether *V. cholerae* from water samples might possess cholera toxin gene. The detection of *V. cholerae* had brought the concern that these water sample might have the possible risk to public. In this study, we could not detect any specific (V.O1 and V.O139) and *ctxA* gen of *V. cholerae,* only detect 17 of NAG carried *ToxR* in 64 water samples in 4 months of a year (from September to December/2012). It is too short time for analysis of the results. The water samples from enviroment need to be collect every month during a year for *V. cholerae* ecology research. Checking the temperature and the concentration of salt of the water in where collecting samples every month need to be done. It will be useful to see the relation between these factor with the number of NAG in enviroment.

The combined of MPN-PCR method used in proved that is effectiveness for the detection of *V. cholerae* using specific primers as long as its density in the sample compared with identification by conventional plating and biochemical test. With MPN-PCR , isolation and enumeration of density can carried out directly. *V. cholerae* O1 and O139 were not detected by m-PCR and DFA methods from all samples. Seventeen of NAG (non-Aglutination) were detected carried ToxR positive.

In this study could not detect *V. cholerae* in surface water samples by DFA (direct fluorescent antibody) assay. Although detection of *V. cholerae* by fluorescent antibody staining has been shown to be highly sensitive in detecting both culturable and non-culturable organisms.

3.12. Conclusions

a) The introduction of MPN-mPCR method is effective for the detection of *V. cholerae* compared with identification by conventional method. Using MPN-mPCR , isolation and enumeration of density can carried out easily and rapidly.

b) *V. cholerae* O1 and O139 were not detected by MPN-mPCR and DFA methods from all samples.

c) NAG with ToxR positive were detected in 17 out of 64 water samples .

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Annex

MPN table for a three-replicate design from FDA's Bacterial Analytical Manual

Positive Tubes

0.1	0.01	0.001	MPN	95% Confidence Range
2	2	0	21	4.5-42
2	2	1	28	8.7-94
2	2	2	35	8.7-94
2	3	0	29	8.7-94
2	3	1	36	8.7-94
3	0	0	23	4.6-94
3	0	1	38	8.7-110
3	0	2	64	17-180
3	1	0	43	9-180
3	1	1	75	17-200
3	1	2	120	37-420

3	1	3	160	40-420
3	2	0	93	18-420
3	2	1	150	37-420
3	2	2	210	40-430
3	2	3	290	90-1000
3	3	0	240	42-1000
3	3	1	460	90-2000
3	3	2	1100	180-4100
3	3	3	>1100	420-4000

3.14. Acknowledgement

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Research 4

4.1 Project title: The basic and clinical study on Histoplasmosis in Vietnam

4.2 General objectives: To investigate the trend of histoplasmosis in clinical and environmental samples in order to elucidate the infectious source in Vietnam.

4.3 Specific Objectives:

1st year

- To improve laboratory capacity on diagnose of histoplasmosis by different methods in Vietnam
- To develop a rapid diagnostic technique for histoplasmosis in Vietnam

2nd year

- To identify the proportion of Histoplasmosis among the patients with lung infection in Vietnam.
- To initially detect *Histoplasma* in the environmental samples.
- To develop a collaborative network for epidemiological study of Histoplasmosis in Vietnam and integrate this network in the overall collaborative network of Southeast Asia.

3rd year

- To disseminate the results to the clinical laboratories in the hospitals, Ministry of Health and the other public health offices in Vietnam.
- To investigate a surveillance of histoplasmosis and other fungal diseases in Vietnam

4.4 Name of Researchers

Promotor: Prof. Tran Hien NGUYEN - National Institute of Hygiene and Epidemiology, Hanoi, Vienam (NIHE)

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4.5 Affiliation

National Institute of Hygiene and Epidemiology, Vietnam.
National Institute of Infectious Diseases, Japan.

4.6 Sub-project title

Evaluation of Histoplasmosis among the patients with lung infection and investigation of the transmission route.

4.7 Summary

Histoplasmosis, a fungal infection caused by *Histoplasma capsulatum* (*H.capsulatum*), has a worldwide distribution and the incidence is about 1 reported case per 100.000 populations (in reported national). Histoplasmosis, which can be acquired from soil contaminated with bird/bat or chicken droppings. The most common clinical presentation is asymptomatic. However, the symptoms of acute or epidemic histoplasmosis are high fever, cough, and asthenia. High risk groups include immunocompromised persons (e.g., persons with cancer, transplant recipients, persons with HIV infection). A proportion of Histoplasmosis has been identified from lung infected patients by an indirect ELISA (HistoplasmaDxSelect™ kit). 40.7% patients were presented anti-*H.capsulatum* antibodies and among them 54.7% were patients with HIV. There were two Histoplamosis case which has detected by nested PCR. However, the soil samples contaminated with chicken droppings have not been identified the presentation of fungal spores. Therefore, the transmission route of the disease is still a challenge and need to be demonstrated in further studies.
Keywords: Histoplasmosis, lung infected patient, ELISA, serum, PCR

4.8 Purposes

Histoplasmosis is a fungal disease caused by infection with *Histoplasma capsulatum (H.capsulatum)*. It took decades to prove that *Histoplasma capsulatum* is a dimorphic fungus, that histoplasmosis is primarily a pulmonary disease, and that the environmental reservoir is soil. Histoplasmosis, which can be acquired from soil contaminated with bird,bat or chicken droppings. Human infection occurs when airborne spores of *H.capsulatum* are inhaled. Histoplasmosis has characteristics of granulomatous disease and primarily affects the lungs and the immune system. The severity of the disease depends on the intensive of exposure, on the number of spores inhaled and on the immune status of the host. The symptoms of acute or epidemic histoplasmosis are high fever, cough, and asthenia. The patient can develop severe pulmonary disease that can lead to respiratory failure and even death. Histoplasmmosis also presents as an opportunistic infection in individuals with serious in underlying disease, including AIDS.

The diagnosis of Histoplasmosis is based on techniques for mycological examination, histological examination, immunological tests and clinical history. A number of excellent laboratory methods are available such as histology, serology, EIA antigen, molecular methods…. However, the fungus is still not easily detected in organic secretions.

H.capsulatum occurs most commonly in North America and Central America, but the organism exists in many diverse areas around the world. Cases have also been reported in the following Asian countries: India, Indonesia, Malaysia, Thailand, Singapore, Vietnam and Japan. In these areas, however, the endemicity of the disease is not well studied, at present. In addition, the clinicians do not consider histoplamosis as a possible cause of acute respiratory or influenza –like illness in travelers returning from areas in which histoplasmosis is endemic and this may contribute to under diagnosis. In Vietnam, histoplasmosis is still under reported because the researchers are inexperienced for detection of histoplasmosis. Physicians, therefore, need to be aware of clinical syndromes and take advantage of the epidemiological clues, such as the activities or occupations that expose the patient to sites contaminated with bat/bird or chicken droppings. In the previous study, several techniques for detection of Histoplasmosis have been successful applied in Vietnam. Therefore, a really situation of Histosplamosis should be identified in this country. In order to find out the trend of the disease, in addition, the resource of fungus is also need to be demonstrated. In the present study, we used the ELISA for detection of antibodies in sera from lung infected patients to identify the proportion of Histoplasmosis. We also tried to establish a relation between environmental reservoirs with the disease in Vietnam.

Objectives

The objective of the study is to identify the proportion of Histoplasmosis among the patients with lung infection in Vietnam and detect *Histoplasma* in the environmental samples in order to investigate the transmission route of *H.capsultum* infection in Vietnam.

4.9 Methods

Sample collection

In five months (from August, 2012 to end of January 2013), the study continued in the Infectious Department, Bach Mai hospital, and 103 Military Hospital. All the lung infected patients combine with HIV positive and/or tuberculosis negative have been selected. Then, bronchial washings and blood from patients have been collected by the nurses and doctors at the hospitals. As environmental sample, soil samples contaminated with chicken droppings around people living areas in Hanoi, BacGiang have been collected by NIHE researcher.

All samples were transported in a safety/cool box to the laboratory and stored at -30^0C until analyzed.

Information on disease situation, history of disease, anti-fungi drug use, place to collect environmental samples has been gathered/recorded in a set of questionnaire by the doctor/researcher after signed informed consent form.

Microbiological methods

All samples were processed and analyzed following the *H.capsulatum* standard operating procedures of National Institute of Infectious Diseases, Tokyo, Japan (NIID).

Briefly, a suspected/infected sample was cultured on BHI agar with 1% glucose and/or 5% sheep blood agar (culture of *H. capsulatum* represents a severe biohazard to laboratory personnel and must be handled with extreme caution in an appropriate pathogen handling cabinet). The suspected colonies will be identified by microscopy and therefore kept at room temperature during one month for further analysis.

Preparation of DNA from clinical and environmental samples have been performed according in house-protocol of NIID (showed in flowchart 1) and the manufacture's instruction of QIAamp® DNA mini kit and PowerSoil™ DNA Isolation kit (MOBIO).

Flowchart 1. In-house protocol for extraction of DNA

Serum 100 µl + lysis buffer 100 µl
↓
Proteinase K (finalconc. 60 µg/ml)
↓
55°C, 60 min
↓
95°C,10 min
↓
Cool down

The template DNA was analyzed by nested PCR for *H.capsulatum* using a set of primer that specifically amplify a DNA coding for a specific portion of *H.capsulatum* M antigen gene (see detail in table 1).

Table 1. Primers using for *H.capsulatum* PCR reaction

Primers	Target gene	Sequence	Lengh of amplicon (bp)
Msp1F	M antigen	5- ACA AGA GAC GAC GGT AGC TTC ACG-3	318bp
Msp2R	M antigen	5- ACC AGC GGC CAT AAG GAC GTC-3	
Msp2F	M antigen	5- CGG GCC GCG TTT AAC AGC GCC-3	269 bp
Msp3R	M antigen	5- ATA AGG ACG TCA CGA AGG GC-3	

DNA amplification procedure

Each 25µl reaction mixture contained 5 µl of the template DNA, 0.5 µl of each primer (50µM/L) and 0.125µl of *Taq* (Takara Ex Taq), 12.5µl 10X PCR buffer, 2.5µl dNTPs and water for a final volume of 25 µl. Amplifications were carried out in a MyCycle™ PCR system (Bio Rad). The following PCR cycle was used: 1x 95^0C for 5min; 40 x (94^0C for 1min followed by 60^0C for 1min and 72^0C for 1min); 1 x 72^0C for 5 min; cool to 4^0C. Ten microliters of PCR product were directly loaded onto 1,5% (w/v) agarose gel for detection of PCR products and DNA fragments separated at 100V for 45min before staining with SYBR safe DNA stain.

Blood samples were also performed by serological test following the manufacture's instruction of HistoplasmaDxSelect™ kit. The test is an indirect ELISA, the polystyrene microwells are coated with inactivated histoplasma antigen. Diluted serum samples and controls are incubated in the wells to allow anti-*histoplasma* antibodies to react with antigen. Nonspecific reactants are removed by washing and peroxidase-conjugated anti-human bound to the antigen. Excess conjugate is removed by washing. Enzyme substrate and chromogen are added, and the color is allowed to develop. After adding the stop reagent, the resultant color change is quantified by spectrophotomeric reading of optical density (OD). ELISA results are compared with the reference cut-off OD readings by an ELISA reading machine.

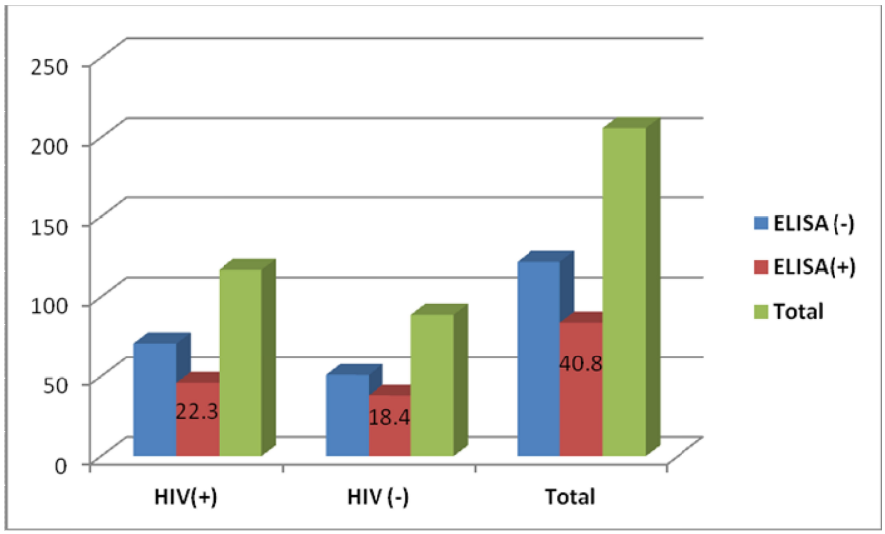
4.10 Results

Up to now, 206 serum samples and 56 bronchial washings have been collected from lung infection patients in the hospitals. Among the serum samples 117 are from HIV positive patients with tuberculosis negative. The other patients are tuberculosis and/or HIV negative patients. At the same time, 120 soil samples contaminated with chicken dropping were collected in the people living areas in Hanoi (including Ha Dong, Dong Anh), Bac Giang.

Fifty six bronchial washing samples were cultured on Brain Heart Infusion (BHI) agar with 1% glucose and BHI containing 5% horse blood and incubated at 37^0C/30^0C for at least one month. Among them 52 samples were negative. The others are still negative, at present.

Serum samples from 206 patients were tested for antibody reactivity by ELISA. Positive ELSIA results were obtained in 84 (40.8%) samples and twenty of these had very high ELISA titer (Fig. 1). These patients have been asked to provide sputum or bronchial washing samples (if possible) to perform a nested PCR. Among them, three patients have provided bronchial washing samples.

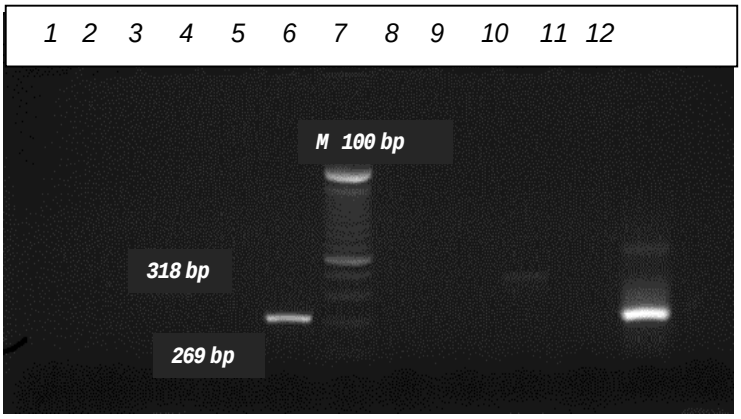
Figure 1. ELISA results of lung infected patients by HistoplasmaDxSelect™ kit



We have already collected the soil samples in the tuberculosis negative patient's house (who had very high ELISA titer) in Bac Giang.

All clinical and environmental samples were negative by the nested PCR. However, we have some clinical samples were positive with the first PCR using primers *Msp* 1F and *Msp* 2R encoding M antigen genes (Fig. 2).

Figure 2. The first PCR results of lung infected patients



Lane 1, 2, 3: samples Lane 4, 12: Negative control
Lane 7-10: samples Lane: 5, 11: positive control
Lane 6: Marker 100bp (Invitrogen)

4.11 Discussion

The incidence of Histoplamosis is wordwide and the infection with *H.capsulatum* occurs during day-to-day activities in highly endemic areas. Pulmonary infection is the primary manifestation of histoplasmosis, varying from mild pneumonitis to severe acute respiratory distress syndrome. Although the definitive diagnosis of histoplasmosis requires the identification of *H. capsulatum* in infected tissue, serological diagnosis can facilitate and provide a rapid identification of the fungus since recovery of the etiological agent is time consuming. In this study, we used HistoplasmaDxSelect™ ELISA kit. The assay utilizes inactivated purified *histoplasma* antigen for the qualitative detection of antibodies to *Histoplasma capsulatum* var. *capsulatum* in human serum. The test is faster than culture, but it has some limitations, including misleading positive results for patients with other diseases caused by microbes that cross-react with *H. capsulatum* and/or the titer of specific antibodies against *histoplasma* remains high for months or even years after primary infection. Otherwise, false negative results are to low antibody titers can be observed in immunocompromised patients with active infection. Therefore, the commercial kit is available for the screening and evaluation of the prevalence of Histoplamosis, only. Our result showed, 40.8% of lung infected patients were positive with ELISA is very high to compare with other Asia countries such as Japan, Thailand. Among them 46/84 (54.7%) were patients with AIDS. The result could be recommended to the clinical doctors, especially in Vietnam – the area in which Hisptoplasmosis is not considered.

In the previous study, we have been applied a nested PCR with the specific primers for detection of *H. capsulatum* in clinical samples and environmental samples such as tissues, body fluids, soil. The design of the primers is based on M antigen genes and the high sensitivity and specificity (it could detect at least 10 fg *H. capsulatum* DNA). In the present work, two clinical samples were negative with the nested PCR. Both of them were also positive with Histoplasma by ELISA. The further analysis should be done by sequence method and compared with the other clinical samples from different areas. Otherwise, the soil samples that we collected from patient's houses and public areas were negative. The reason

might be the patients are not often living in their place and the sample size in the public areas is small. We also have to think about the situation of the clinical ward and surrounding areas in the hospital. It could be one of risk factors for transmission of disease in Vietnam, at present. In addition, to describe the overall situation of histoplasmosis in Vietnam, the sampling work should be closely collaborated with the epidemiology department.

In conclusion, the proportion of Histoplasmosis in lung infected patients is very high (40.8%). It is the first report on Histoplasmosis prevalence in high risk group in Vietnam, especially HIV patients. The transmission route of the disease is still a challenge and need to be demonstrated in further studies.

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Research 5

5.1 Project title: Establishment of laboratory diagnosis for leptospirosis and investigation of prevalence of leptospirosis among patients with fever of unknown origin in northern area of Vietnam.

5.2 General objectives:

To investigate the prevalence of leptospirosis in Vietnam

To describe the transmission routes

5.3 Specific Objectives:

1st year

To perform a differential diagnosis of *Leptospira* from samples in Vietnam using the following techniques: microscopic agglutination test (MAT; gold standard method), recombinant protein (LigA)-based IgM ELISA, whole cell-based IgM ELISA and molecular methods (*i.g*. PCR, loop-mediated isothermal amplification – LAMP).

2nd year

To determine the proportion of leptospirosis among patients with fever of unknown origin in several general hospitals in Northern of Vietnam by using a suitable ELISA method

To detect *Leptospira* in the suspected animals and contaminated environmental samples by culture and molecular methods.

3rd year

To investigate the prevalence of *leptospira* infection in Northern areas of Vietnam

To evaluate the relation between human, animal, and environment in order to determine the transmission routes of leptospirosis in northern Vietnam.

5.4 Name of Researchers

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- Nobuo KOIZUMI, PhD, Makoto OHNISHI PhD - Department of Bacteriology - National Institute of Infectious Diseases

5.5 Affiliation

National Institute of Hygiene and Epidemiology, Vietnam.

National Institute of Infectious Diseases, Japan.

5.6 Sub-project title:

Using PCR and ELISA for evaluation of leptospirosis among patients with fever of unknown origin and a suspected transmission route of the infection

5.7 Summary

Leptospirosis is a transmissible disease of animals and humans caused by infection with any of the pathogenic members of the genus *leptospira*. Leptospirosis infection was defined the criteria based on (non-hepatitis) jaundice disease, (non-dengue) hemorrhagic disease, and (non-malaria) febrile disease and confirmed by laboratory tests. Laboratory diagnosis of leptospirosis is designed to detect anti-*leptospiral* antibodies (ELISA, MAT) and to detect *leptospiral* antigens, or *leptospiral* DNA (PCRs) in animals and humans. The

particular testing regimen selected depends on the purpose of testing and on the tests or expertise available in the area. Vietnam has been considered endemic for leptospirosis but information on the prevalence of disease is lacking except Mekong Delta region. To investigate a proportion of leptospirosis, especially in origin unknown fever patients in Northern of Vietnam, we used ELISA with the recombinant protein (LigA)-based IgM for detection of *leptospiral* antibodies. In addition, the *flaB*-nested PCR using mixture specific primers in the 16S region of ribosomal RNA have performed for detection of *leptospiral* DNA from blood, urine and tissue samples. The IgM-ELISA results showed 9.8% of origin unkown fever were positive and 62.4% of them are farmers and worker in the meat market. Using *flaB*-nested PCR, DNA *leptospira* were idenitfied in three patients with very high IgM ELISA titer. However, there was no evidence of the prensence of *leptospiral* DNA in animals/environmental samples. With this PCR, we should be concerned on the inhibitory factors of samples and/or amount of samples in the further studies.

Keywords: leptospirosis, nested PCR, IgM, ELISA, unknown fever patients

5.8 Purposes

Leptospirosis is a zoonosis with preference for warmer climates, and caused by pathogenic *leptospiral* species. Rats and other rodents are the most important sources for human infection. An infected animal can remain asymtomatic and continued to shed infectious organisms in the urine for its entire lifetime. Humans usually become infected though contact with urine contaminated soil or water, with infected animal tissue, or from rat bites. Thus, farmers, sewer workers, miners, fishermen, and meat workers have traditionally been at greatest risk for infection. Recently, however, many leptospirosis cases have been reported with the transmission of water in the swimming pool. It could be changed the modes of human interaction with the environment. Leptospirosis infection was defined the criteria based on (non-hepatitis) jaundice disease, (non-dengue) hemorrhagic disease, and (non-malaria) febrile disease and confirmed by laboratory tests such as serology, culture, and molecular methods.

Since the 1930s Vietnam is known to be endemic, with the first case of Leptospirosis in Northern Vietnam described in 1937. High rate of seropositivity against *leptospira* in the Mekong delta has been observed in previously, ranging from 10-30% [Van CT. et al 1998]. Another study in Thanh Hoa – Northern of Vietnam, showed that one of the serovars included in the major pathogenic species has been isolated in this area (unpublished data). However, the published information did not mention on the risk factors and a reservoir animal survey. In addition, in Vietnam, animal housing and feeding conditions are poor, so the disease could be easily transmitted from animal to animal and from animal to human. As predicted by the Center for Preventive Medicine of Hanoi, the disease can thrive during the rainy season because sanitation is seriously degraded and rodent rapidly reproduces. Currently, the risk of an outbreak of leptospirosis in residential areas, slum areas and rail/car stations is very large due to poor sanitation, frequent and prolonged flood.

Serology is most frequently used diagnostic approach for leptospirosis. Enzyme-linked immunosorbent assays to detect *leptospira*-specific antibodies have recommended and widely used. These tests have the advantage of providing rapid results without the need for culture and microscopic agglutination test (MAT) facilities. Detection of immunoglobulin M (IgM) by ELISA was performed to diagnosis acute leptospirosis, especially screening for unknown fever patients. In addition, the PCR is a sensitive, specific, and rapid technique which has been successfully applied to the detection of several pathogens in a variety of specimens, including serum, urine, feces… A nested PCR using specific primers has been reported to demonstrate *leptospira* in urine, serum samples from patients with leptospirosis at in different stages of the infection. The method is also useful for identification of bacteria from reservoir animal and environment.

In this present project, we investigate the proportion of leptospirosis among patients with fever of unknown origin by using a suitable ELISA method and initially identify *leptospira* in the animals and contaminated environmental samples by a *flaB*-nested PCR.

5.9 Methods

Sample collection

From August, 2012 to end of January 2013, samples were obtained from origin unknown fever patients admitted to the BachMai and Military 103 hospital, Hanoi, ThanhHoa General hospital, with history and clinical manifestation suggestive of leptospirosis, including fever, headache, myalgia with any of the following: conjunctival suffusion/haemorrhage; meningeal irritation; hemorrhage – intestinal bleeding, lung bleeding or purpuric rash; cardiac arrhythmias/failure; jaundice. The blood samples for serology and urine for PCR were collected on the day of admission.

Urine samples from animals such as pigs and cattle were collected in Yen Dinh and NhuThanh sub-urban, ThanhHoa. The kidney tissue samples from animals (dog/rat) have also been collected. At the same area, the contaminated soils were taken by the local researchers.

All samples were transported in a safety/cool box to the laboratory and stored at -30^{0}C until analysis.

Information on the history of exposure to infected animals/environment contaminated with animal urine, age, gender, time to contact with risk factors, social – economic, education has been recorded in a comprehensive set of questionnaire by NIHE researchers.

Microbiological methods

We performed an ELISA with the recombinant protein (LigA)-based IgM which has evaluated in the previous study on by the NIID researcher group.

Leptospira DNA was analyzed in the same sample by nested PCR (*flaB*-nested PCR).

DNA extraction from blood and urine: blood and urine were centrifuged with 100×*g* at 4^{0}C for 5min in order to remove red blood cells or, cell debris and protein precipitates. The supernatant was transferred to a new tube and centrifuged at13.000×*g* at 4^{0}C for 20min. DNA was extracted from the pellet by using DNAeasy Tissue Kit (QIAamp®, Germany) according to manufacturer's instructions.

In order to amplify DNA in blood and urine samples, L-*flaB*1/M-L-*flaB*2 primers were used as described by NIID's group. The reaction with L-*flB* F1 5'-TGTGCACAAGACGATGAAAGC -3' (23 Nu) and L-*flB* R1 5'-AACATTGCCGTACCACTCTG -3' (22 Nu) primers consisted of 94^{0}C for 1min, 25 cycles at 94^{0}C for 10s, 50^{0}C for 30s, 72^{0}C for 1min, and 72^{0}C for five additional minutes. The reaction with M-L-*flaB* F25'-TGTGCACAAGACGATGAAAGC-3' (21 Nu) and M-L-*flaB*R25'- AACATTGCCGTACCACTC TG -3' (20 Nu) primers consisted of 94^{0}C for 1min, 30 cycles at 94^{0}C for 10s, 50^{0}C for 30s, 72^{0}C for 50s, and 72^{0}C for seven additional minutes. These primers correspond to a conserved sequence in the 16S region of the ribosomal RNA.

Eight microliters of PCR product were directly loaded onto 1,5%- 2% (w/v) agarose gel for detection of PCR products and DNA fragments separated at 100V for 20min before staining with SYBR safe DNA stain.

5.10 Results

At present, we collected 285 blood samples from unknown fever patients in the hospital. Three of them are patients from ThanhHoa province. The blood samples were centrifuged and divided in two sterilized tubes. At the same time, 40 urine samples and 95 kidney tissues from dog and rat as well as 70 soil contaminated with animal urine have been collected in ThanhHoa province.

All serum samples were tested by ELISA with the recombinant protein (LigA)-based IgM. Twenty eight patients of 285 (9.8%) presented acute leptospirosis infection with a positive ELISA results (Fig.1). Among them, 18 patients are farmers and worker in the meat market, and these others are not in the occupational risk group. Three patients came from

ThanhHoa were negative by IgM-ELISA (but they were positive with IgG-ELISA by IVD Leptospira-ELISA microwells kit).

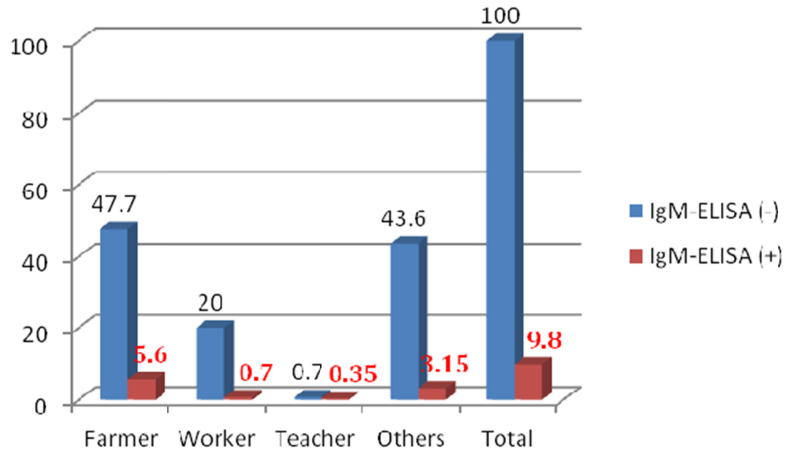

Fig.1. IgM-ELISA with recombinant antigen LigA results from serum samples of patients

DNA has been extracted from the serum, urine, tissue samples and analyzed by nested PCR for the specific *leptospira* genes. Three patients were presented *leptospira* DNA in serum sample (fig.2). There other samples were all negative.

During the study period, we cultured the tissues kidney samples from dogs and rat. All of samples were negative by culture, at present. We have also cultured 24 *leptospira* strains that were kindly provided from Ho Chi Minh Pasteur Institute for MAT testing and preparing positive control. The culture tubes are daily checked and re-cultured to a fresh medium every 7 days.

5.11 Discussion

Many ELISAs have been developed using a number of different antigen preparations. Detection of IgM antibodies by ELISA is now widely used in the diagnosis of leptospirosis in specialized laboratories. It has both high sensitivity and specificity if the blood sample is taken several days after the typical symptoms are first noted, when the IgM antibodies have had time to develop. In addition, the IgM-ELISA assay was more rapid and sensitive than MAT for detection of cases early in the acute illness. In the previous study, ELISA with the recombinant protein (LigA)-based IgM was presented a higher rate of IgM positive results than ELISA with the recombinant protein (LigA)-based, respectively 10%, 7.2%. Therefore, in this study, we used IgM-ELISA with the recombinant protein (LigA) to identify the proportion of leptospirosis in origin unknown fever patients (severe, acute illness stage).The ELISA results were 9.8% (28/285 cases) with 62.4% positive cases were farmers and worker in the meat market (18/28). It could be suggested the infection were closely related to their works. However, we were not able to obtain more detailed information on occupations of all patients. In addition, they are not resident people in Hanoi. In the future, therefore, it will important to identify other the risky activities than the occupational risk group in order to prevent and control the infection. The main reason for seeking an early diagnosis of leptospirosis is to facilitate appropriate treatment, particularly if the choice of appropriate antibiotic treatment is to be guided by diagnostic test results. However, in Vietnam, the patients can buy antibiotics and treat themselves before they are admitted to the hospital. It could be given a false negative result, especially for PCR method. Since the early symptoms of letospirosis are often regards as non-specific, in many poorer rural populations, where medical attention may be both difficult to obtain and costly, it is less likely acute-phase samples will be taken. Therefore, it is not easy to select a real acute stage of the patients. In addition, the subjects in this study were selected with the main criteria as origin unknown fever. Moreover, a limitation to use of single serum samples for sero-diagnosis is the persistence of IgM antibodies. Anti-leptospiral IgM antibodies are decline after short time. Thus far, a single IgM-positive sample taken during an acute illness with symptoms suggestive of leptospirosis of presumptive evidence of

infection, but this finding requires confirmation by further testing. However, although microscopic agglutination test (MAT) is definitive diagnosis of infection, but IgM-ELISA is the first choice for the laboratorytesting.

Leptospiral DNA can be demonstrated in tissues, body fluid, soil samples using a variety of assays based on the polymerase chain reaction (PCR). A number of primers sets for the conduct of PCR assays have been described with some primers only specific for the genus leptospira and others designed to identify only pathogenic species. PCR assays can be quite sensitive, but lack of specificity (i.e. false positive results) can be a problem. The contamination of sample or using antibiotic situation can be cause of false negative results. Therefore, quality control of PCR assays used for diagnosis of leptospirosis requires careful attention to laboratory condition and sample processing procedure. The primers used in this study have been confirmed in NIID and given a good sensitivity and specificity. Our nested PCR results showed three serum samples were positive, only. These samples were collected from the patients with very high ELISA titer. However, all tissue/urine and soil samples were negative by nested PCR. It means that, the detection of leptospira DNA from different sources is still a challenge.

In conclusion, the proportion of leptospirosis in origin unknown fever patients in the study hospitals is 9.8% and the ELISA with the recombinant protein (LigA)-based IgM can be used for early diagnosis of leptospirosis, but a positive results will need to confirm by other tests. In this period study, there is no case that presented *leptospira* DNA in animal/enviromental samples in ThanhHoa areas. Thus, we should be concerned on the inhibitory factors of samples and/or amount of samples for PCR methods in the further studies.

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Research 6

6.1 Project title: The improvement of the epidemiological surveillance of Anthrax in Vietnam

6.2 Specific Objectives:

The 1st year

To apply differential diagnostic tools of anthrax in Vietnam.

The 2nd year

To describe the molecular characterization of *B.anthracis* isolates in Vietnam.

To determine the epidemiological characterization of Anthrax disease in Vietnam.

The 3rd year

To identify *B.anthracis* spores in environmental sources in the high risk areas in Vietnam

To compare Vietnamese *B.anthracis* isolate strains with the other Asian countries

To defense a Master thesis and submit an international scientific paper

To increase a strongly network for surveillance of Anthrax in Vietnam and also work in collaboration with other international/national institutions for further studies

6.3 Name of Researchers

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6.5 Sub-project title: Molecular epidemiological study of *B.anthracis* isolated in Vietnam

6.6 Summary

Anthrax is an acute bacterial zoonoses caused by a Gram-positive bacterium, *Bacillus anthracis (B.anthracis)*. Human beings almost invariably contract anthrax from animals. The infection is more clearly by showing the evidence of the transmission sources. Recently, anthrax incidence in humans is frequently occurred in Vietnam, especially in the high mountain areas eg. Hagiang, DienBien, LaiChau, LaoCai. A conventional PCR has used to identify the presence of the virulence genes of bacteria (*pag, cap, Ba813*). In addition, to describe the molecular characterization of *B.anthracis* strains in Vietnam, a multiple-locus variable-number tandem-repeat for 8 loci – MLVA-8 (*vrrA, vrrB1, vrrB2, vrrC,1, vrrC2*, CG3, pXO1, pXO2) has been performed. In this study, three *B.anthracis* strains were isolated from the cutaneous patients in TuanGiao communue, DienBien province. The MLVA-8 results showed that, six *B.athracis* strains (including three strains from HaGiang, LaiChau, DienBien in 2009 and three new strains in DienBien) were same number of repeats in the locus and there were no genetic differences associated with the geographical distribution and time of these strains. Our results suggested that MLVA-8 is one of the modern methods for

studying the molecular epidemiology of Vietnamese *B.anthracis* isolates. In addition, to find out the source of the infection, further studies need to detect and isolate *B.anthracis* from environmental samples.

Keywords: *B.anthracis*, zoonosis disease, PCR, MLVA-8, patients

6.7 Purposes

Bacillus anthracis (*B.anthracis*), a spore forming Gram positive bacteria, is the causative agent of anthrax in human and animals. The spores are primarily brought into contact with herbivores through ingestion of contaminated soils. Human infection generally occurs when persons come in contact with anthrax-infected animals, infected animal products or handling of environmental samples.

Anthrax incidence in humans is frequently occurred in Vietnam recent years, especially the mountainous provinces in the North as Laocai, Hagiang, LaiChau, DienBien, with very low living conditions and limited transportation. Recently, 34 clinical cases have been reported in those areas. Most of infected cases have been identified by clinical symptoms only and related to eat infected animals. Otherwise, in these areas, sporadic cutaneous cases had no contact history with infected animals. Spore-forming in the soil is possibly caused of those infected cases. In addition, due to lacking knowledge and instruments, the local researchers or technical staffs working in diagnosis/research laboratories have been exposing frequently with pathogen. The process of transporting infected sample could be affected to community and environment.

A number of molecular methods have been described for the identification of *B.anthracis* from different types of sample. The main characteristic used to distinguish *B.anthracis* from closely related soil –borne *Bacillus* is the presence of two virulence plasmids pXO1 and pXO2. Virulence factor genes *lef*, *cya*, *pag* (pXO1) and cap (pXO2) have been used as markers to detect *B.anthracis* in the suspected samples using the polymerase chain reaction (PCR). PCR detection of *B.anthracis* in infected animal or patient specimens is easy but is difficult in environmental resource e.g. soil. In addition, numerous studies have demonstrated the lack of molecular polymorphism with thin *B.anthracis*. Recently, PCR-based methods have become increasingly important to molecular typing efforts. However, a limitation of many PCR-based approaches is the binary nature of their data, the presence or absence of a marker fragment. In its best application, multiple locus variable-number tandem repeat analysis (MLVA) can discriminate among different *B.anthracis* isolates. In the literature, MLVA using eight loci *vrrC1, vrrC, vrrB1,vrrB2, CG3, pXO1-aat* and *pXO2-at* has been reported. In this study, we used different methods for detection of *B.anthracis* in order to determine molecular characterization of *B.anthracis* isolates and to describe epidemiological aspects of Anthrax in Vietnam.

6.8 Methods

Sample collection

From July, 2012 to end of January 2013, the study was performed in DienBien province. Based on the results of the first year, two sub-urbans (Tuan Giao and Tua Chua) have been continued to collect samples. This work was based on the network between NIHE, PMC and Department of Animal Health in DienBien which has been created in the last study.

According to Anthrax standard operating procedure, the following samples were collected by the local staff: feces, tissues, body fluid, blood, skin from infected animals, patients and environmental samples. Concurrently, at least 5 soil samples were collected from the patient's house and infected animal cages.

All information on disease situation, history of disease (direct contact to infected animal, soil….), age, gender, social economic situation, education, geographic etc...were asked following a comprehensive questionnaire by NIHE researchers as previously done .

All samples were transported in a safety/cool box to the laboratory and stored at -80^0C until analyzed.

Sample preparation

All sample preparation work has conducted under Biosafety laboratory level 3 conditions.

Bacterial strains:

17JB *B.athracis* strains and other *B.anthracis* isolates were kindly provided by National Institute of Hygiene and Epidemiology frozen strains Bank.

Culture and DNA extraction:

B.anthracis 17JB and three isolates (BA1.09, BA2.09, BA3.10) were inoculated into trypsoy broth and incubated at 37^0C overnight without shaking. Then isolates were grown on 5% sheep blood agar and (BA) and nutrient agar (NA) for selecting purified colonies of *B.anthracis* following overnight and incubated overnight at 37^0C. Purified colonies from each plate were picked up with an inoculation loop and suspended in 2ml eppendorf tubes containing 500µl of sterilize distilled water. The suspensions were then heated at 100^0C for 15 min. The supernatant was used for DNA extraction using the Biospin Bacteria Genomic DNA Extraction kit (Biospin, Hangzhou, China); extraction was performed according to manufacture's instructions). The DNA templates were used as positive control and for molecular typing purpose.

On the other hand, 40 fresh clinical samples were processed and cultured and/or extracted by using QIAamp® DNA mini kit (according to manufacture's instructions)

60 soil samples were extracted by PowerSoil™ DNA Isolation kit (MOBIO) (according to manufacture's instructions). DNA extracts were stored at minus 20^0C until running PCR.

PCR

Detection of *B.anthracis* by conventional PCR was performed with selected published primers that were reported to efficiently amplify targets located on the chromosome, pXO1 and pXO2 (see detail in table 1).

Table 1. Sequences of DNA oligodeoxy-nucleotides used as primers in the PCRs

Primers	Target gene	Sequence	Length (bp)
PA7 PA6	*pag*	CTACAGGGGATTTATCTATTCC ATTGTTACATGATTATCAGCGG	151 bp
MO11 MO12	*cagC*	ACTCGTTTTTAATCAGCCCG GGTAACCCTTGTCTTTGAAT	264 bp
Ba813 R1 Ba813 R2	*Ba813R*	TTAATTCACTTGCAACTGATGGG AACGATAGCTCCTACATTTGGAG	152 bp

DNA amplification procedure

Each 25µl reaction mixture contained 1 µl of the template DNA,1 µl of each primer (20pmol/L) and 12,5µl of *Taq* PCR master mix (QIAgen, Germany) and enough water for a final volume of 25 µl. Amplifications were carried out in a MyCycle™ PCR system (Bio Rad). The following PCR cycle was used: 1x 94^0C for 5min; 30 x (94^0C for 30s followed by 55^0C for 30 s and 72^0C for 55s); 1 x 72^0C for 5 min; cool to 4^0C. Ten microliters of PCR product were directly loaded onto 1,5% (w/v) agarose gel for detection of PCR products and DNA fragments separated at 100V for 45 mins before staining with SYBR safe DNA stain.

MLVA-8

The molecular characterization of *B.anthracis* isolates was analyzed by using Multiple Locus Variable-Number Tandem Repeat 8 loci (see detail in table 2)

Table2. Primer used for MLVA-8

Marker locus	Primers	Primer sequence

vrrA	*vrrA*-f1-fam	CAC AAC TAC CAC CGA TGG CAC A
	vrrA-r1	GCG CGT TTC GTT TGA TTC ATA C
vrrB1	*vrrB1*-f1-fam	ATA GGT GGT TTT CCG CAA GTT ATT C
	vrrB1-r1	GAT GAG TTT GAT AAA GAA TAG CCT GTG
vrrB2	*vrrB2*-f1-fam	CAC AGG CTA TTC TTT ATC AAA CTC ATC
	vrrB2-r1	CCC AAG GTG AAG ATT GTT GTT GA
vrrC1	*vrrC1*-f1-fam	GAA GCA AGA AAG TGA TGT AGT GGA C
	vrrC1-r1	CAT TTC CTC AAG TGC TAC AGG TTC
vrrC2	*vrrC2*-f1-fam	CCA GAA GAA GTG GA ACCT GTA GCA C
	vrrC2-r1	GTC TTT CCA TTA ATC GCG CTC TAT C
CG3	CG3-f1	TGT CGT TTT ACT TCT CTC TCC AAT AC
	CG3-r1	AGT CAT TGT TCT GTA TAA AGG GCA T
pXO1-aat	pXO1-aat-f3-fam	CAA TTT ATT AAC GAT CGA ATT AAG TTC A
	pXO1-aat-r3	TCT AGA ATT AGT TGC TTC ATA ATG GC
pXO2-at	pXO2-at-f1	TCA TCC TCT TTT AAG TCT GT
	pXO2-at-r1	GTG TGA TGA ACT CCG ACG ACA

DNA, 5*ng* in final volume of 50µl with *Taq* DNA polymerase, and 0.1µM of each primer was amplified using a Veriti Thermal Cycle (Life Technologies). The reaction was carried out as following: 94^0C for 2 min; 30 x (94^0C for 30s followed by 52^0C for 30 s and 68^0C for 1 min); 1 x 68^0C for 2 min. Then, PCR products were purified by using PCR genomic purification kit (Invitrogen) and direct sequencing was performed using a 3730 xl DNA analyzer (Life Technologies), a Big Dye Terminator v3.11 Cycle Sequencing Kit and appropriate primers. DNA sequences obtained from the multiple fragments were assembled and edited by GENETYX ver.9 (GENETYX Corp. Tokyo, Japan).

The MLVA-8 results for 6 isolates from Dien Bien, Laichau and HaGiang were analyzed by using MEGA5 software.

6.9 Results

In this period (7 months from July), we have identified 10 suspected human case. Most of the patients were cutaneous disease. Seven of them had acquired infection through contact with animals and the others had no clear exposure information. A total 20 eschar and vesicular fluid samples were collected from patients. At the same time, 20 tissue samples were taken from livestock in the patient's house and market. Sixty soil samples were also collected around the cage of the cow/goat and suspected patient houses in Dien Bien.

The clinical samples were used for both culture and DNA extraction following the standard SOP. Three vesicular fluid specimens were positive by culture. DNA has been extracted from these colonies for analyzing by molecular methods. Three skin specimens were presented *pag* and *cap* genes (Fig. 1).

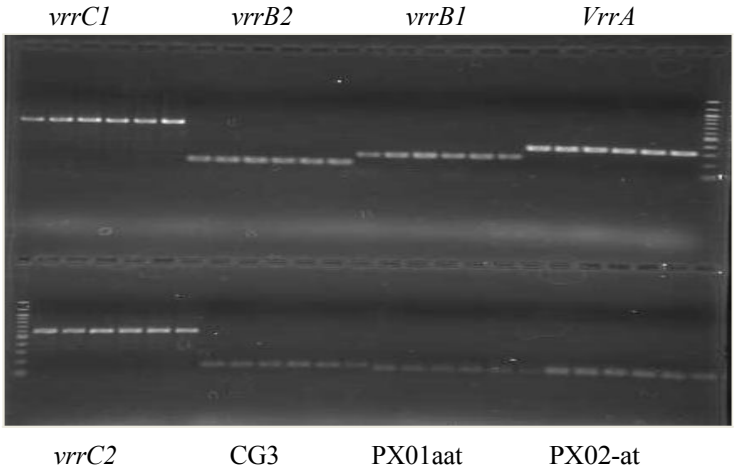
Six *B.anthracis* isolates were analyzed by MLVA-8 (Table 3). Number of tandem repeats in the loci, *vrrA, vrrB1, vrrB2, vrrC1, vrrC2* and CG3 were identical among those isolates. The isolates from LaiChau (BA1.09) and DienBien (BA2.09, BA3.09) had 7 repeats of pXO1 and pXO2 as three new *B.anthracis* isolates in DienBien.

Table 3. List of *B.anthracis* strains of Vietnam with the number of tandem repeats by MLVA-8

Stains no.	Isolated place	Source	No. of tandem repeats by MLVA-8 determined in this study							
			vrrA	*vrrB1*	*vrrB2*	*vrrC1*	*vrrC2*	CG3	pXO1	pXO2
BA1.09	Hagiang	vesicular fluid	4	20	8	57	21	1	7	7
BA2.09	LaiChau	eschar	4	20	8	57	21	1	7	7
BA3.09	DienBien	vesicular fluid	4	20	8	57	21	1	7	7
BA4.11	DienBien	vesicular fluid	4	20	8	57	21	1	7	7
BA5.12	DienBien	eschar	4	20	8	57	21	1	7	7

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***

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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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- Satoshi Inoue
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7.5. Affiliation

– National Institute of Hygiene and Epidemiology, Vietnam.
– 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.

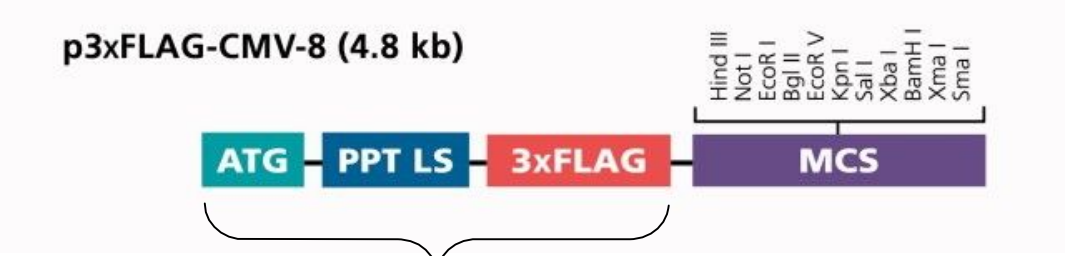
7.9. Methods.

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

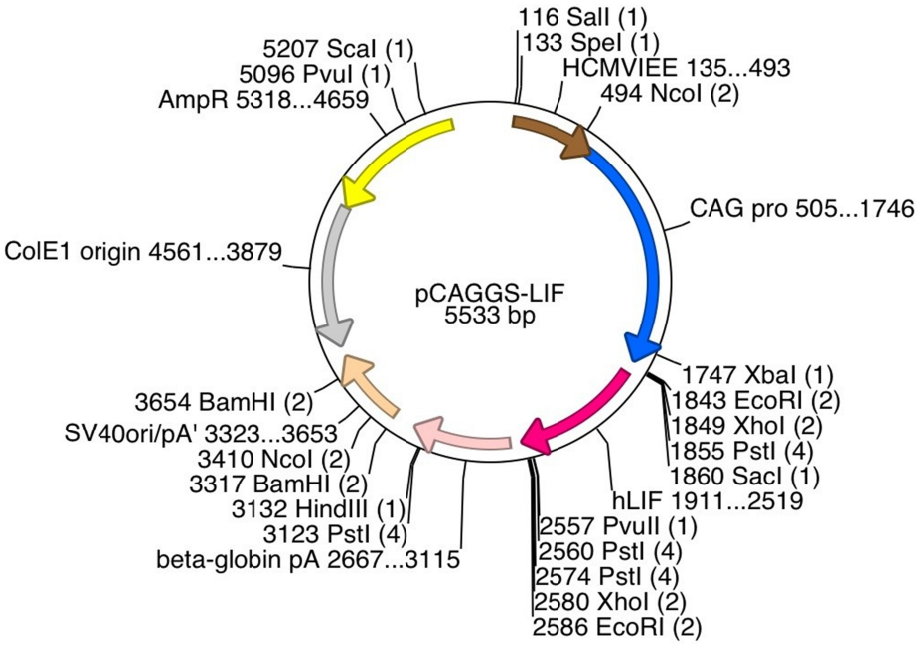
The primers and cloning sites for N gene cloning were designed and determined by GENETX software, version 9 based on the full length sequence of N gene of rabies viruses which were obtained from gene bank.

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^{o}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^{o}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)
- GCGGCCGCgaatcactcgaatacgtcttg Rabies-N 3' primer (including NotI)

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

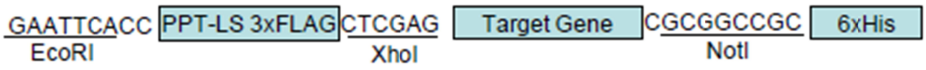
EcoRI and XhoI

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

XhoI and NotI

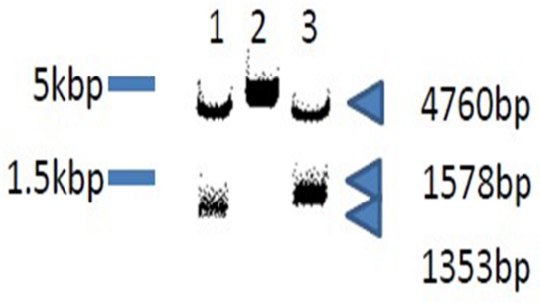
7.10.2. Results of construction of N rabies inserted modified plasmid pCAGGS-P7

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

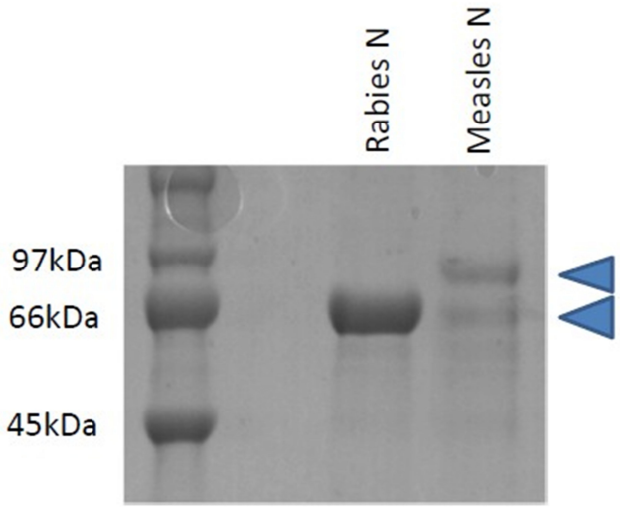
The inserted gene then was checked by Enzyme cutting using by XhoI and NotI, the picture 4 showed the target N rabies gene (lane 1) and Measles N gene (lane 3) were successfully inserted into the pCAGGS-P7. The lane 2 showed the single cut of pCAGGS-P7 inserted Rabies N gene using XhoI.



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

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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	-	+	

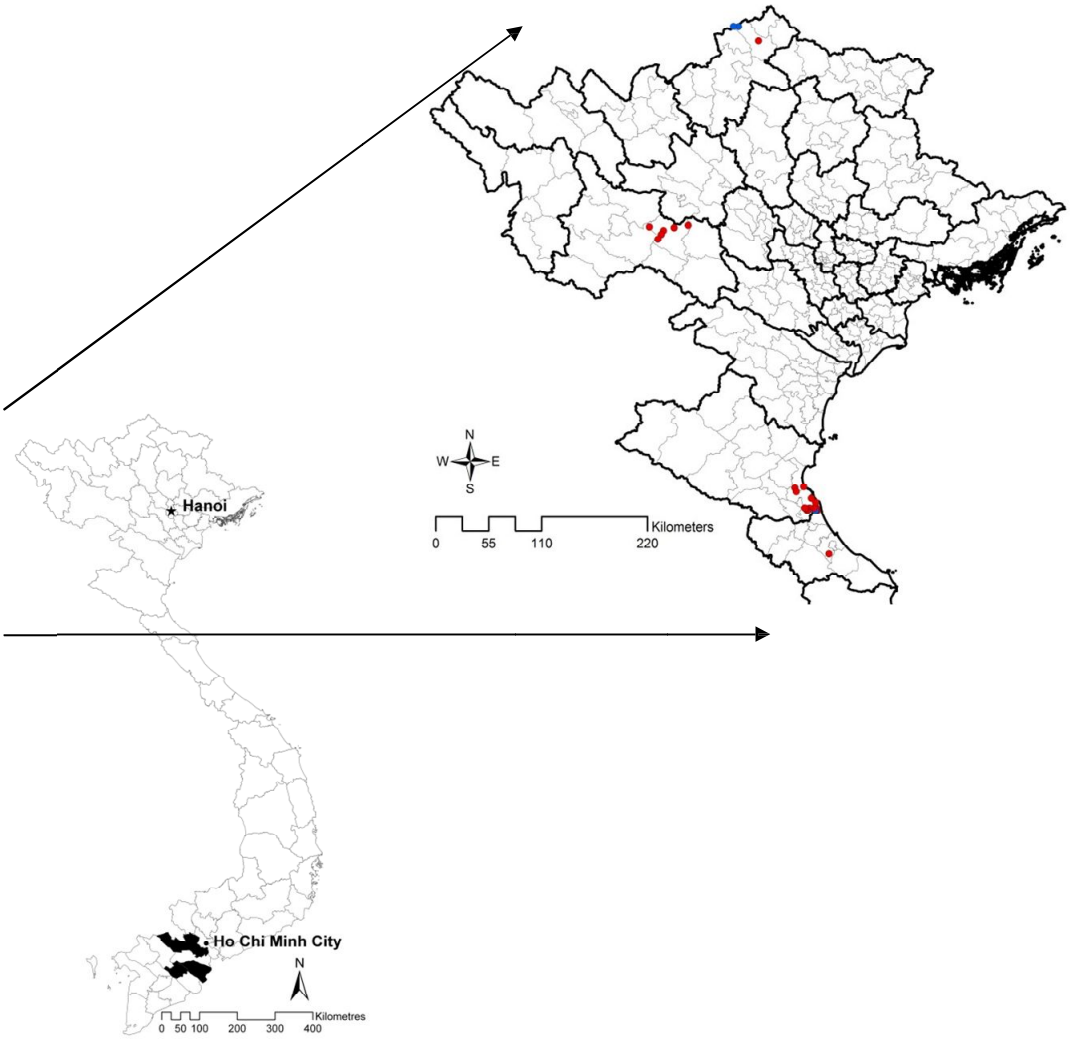

Figure 1: Location of 28 specimens taken in 2010. 25 samples were positive MV (by virus isolation and / or RT-PCR: ● ; 3 samples were negative MV: ●

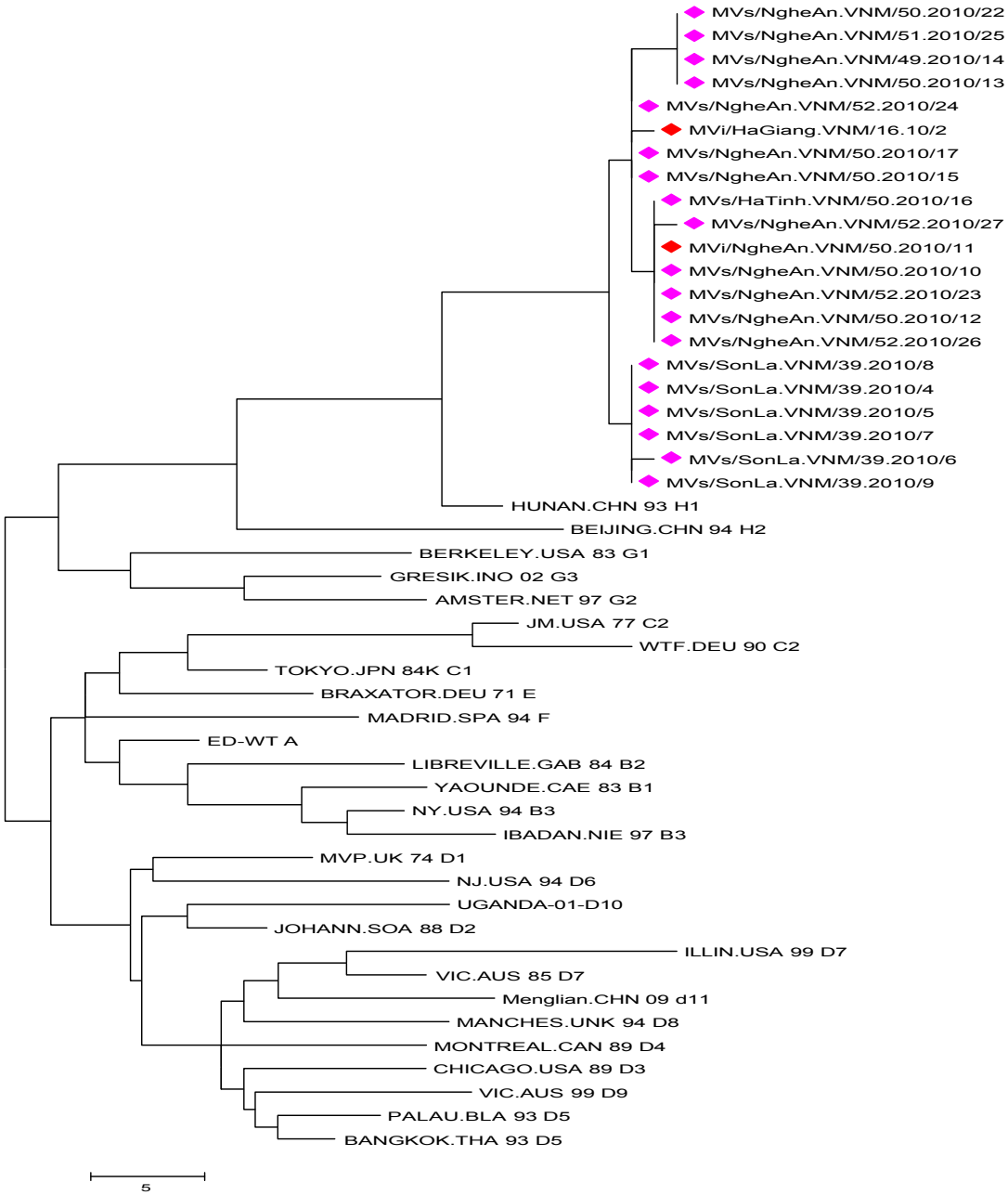

Figure 2. Phylogenetic analysis of the sequences of the nucleoprotein genes (450 nt) of the strains of measles virus in 2010 in the North Viet Nam. The unrooted tree shows sequences from Vietnam viruses compared with World Health Organization (WHO) reference strains for each genotype. MVs, measles virus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

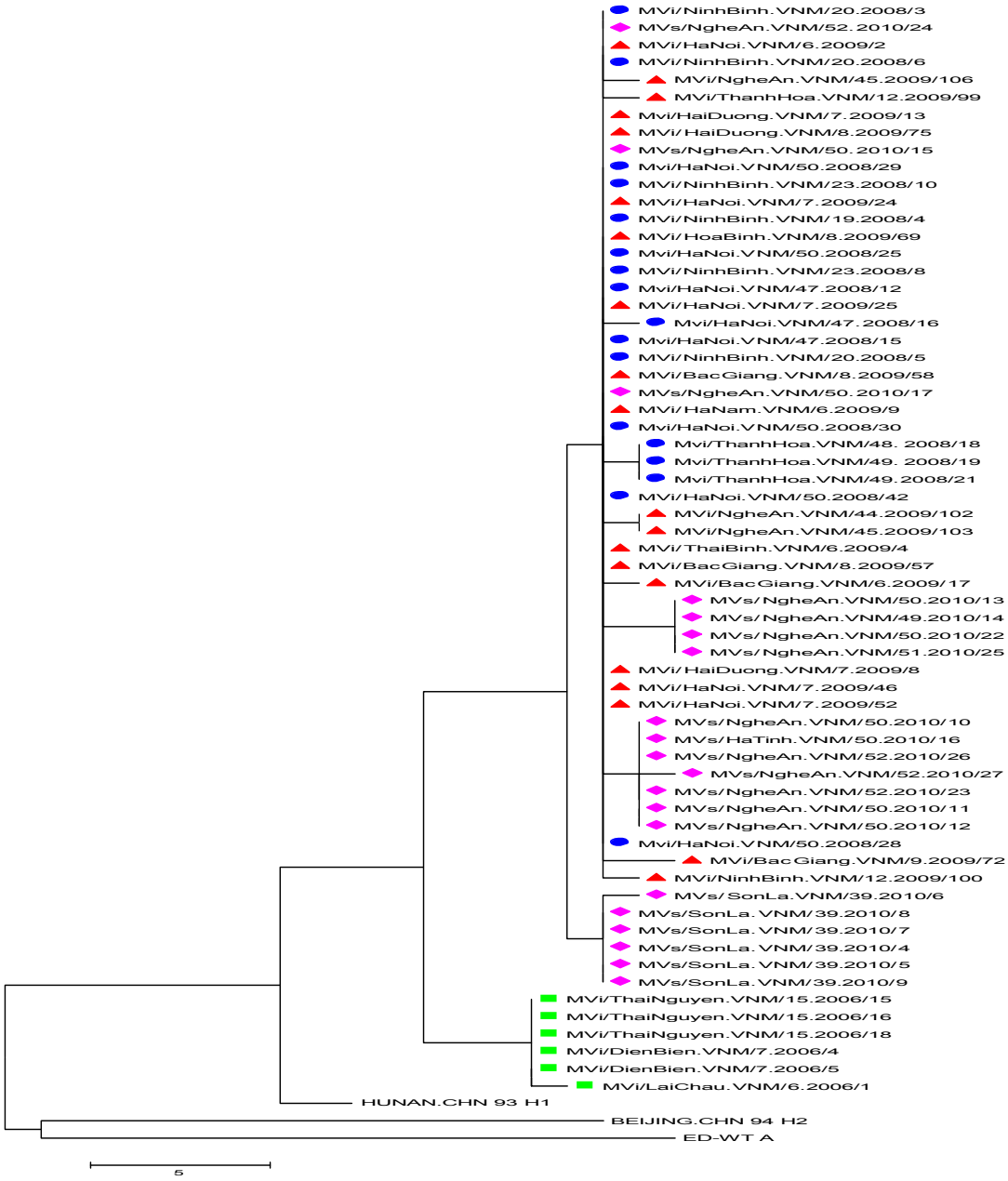

Figure 3. Phylogenetic analysis of the sequences of the nucleoprotein genes (450 nt) of the strains of measles virus from 2006- 2010 in the North Viet Nam and reference strains for H1, H2 and A.
MVs, measles virus sequence from throat swab samples.
MVi, measles virus sequence from isolates.
Sequences 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	-	-	

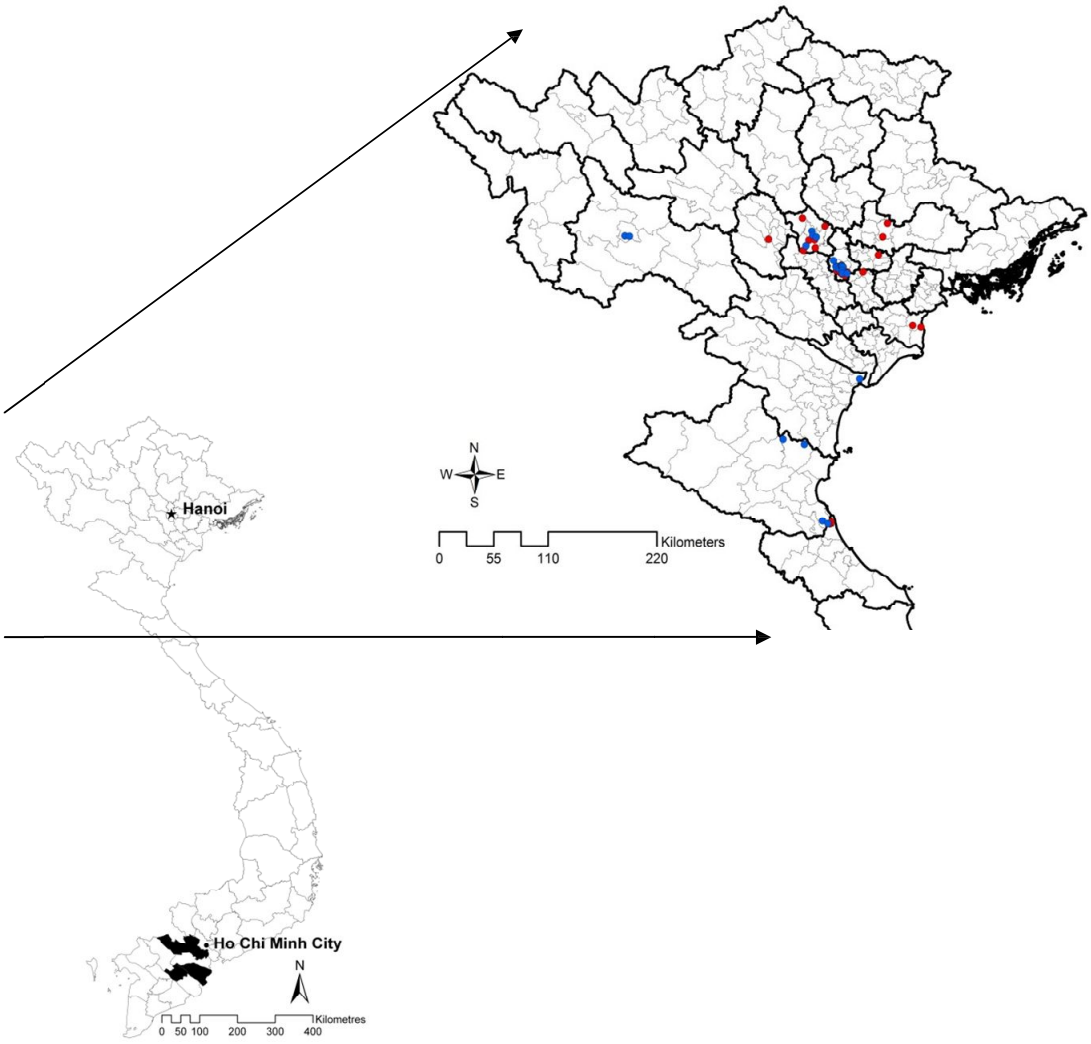

Figure 4: Location of 55 specimens taken in 2011. 31 samples were positive RV (by virus isolation and / or RT-PCR: ●; 24 samples were negative RV: ●

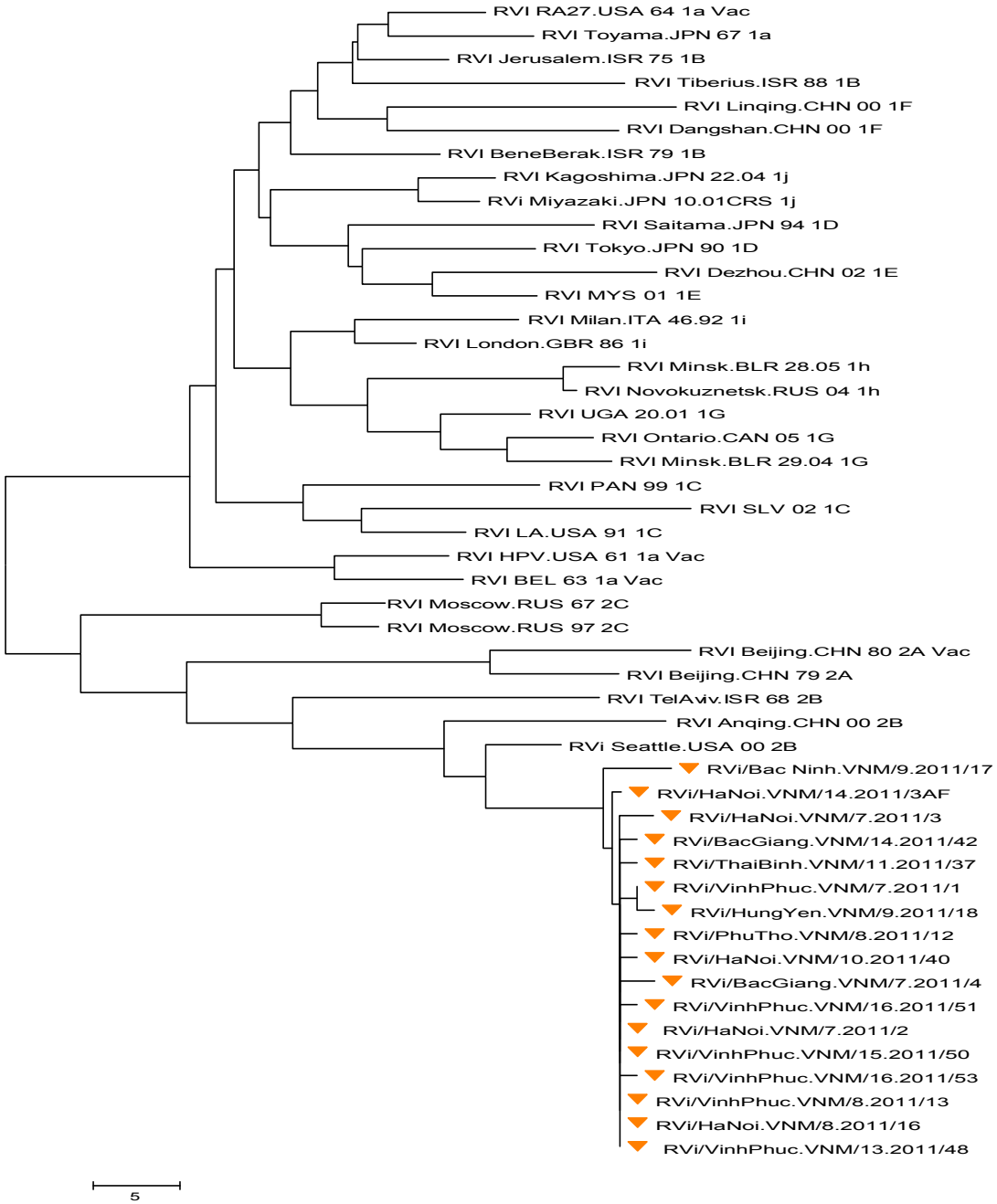

Figure 5. Phylogenetic analysis of the sequences of the E1 gene (739nt) of the strains of rubella virus from 2011. The unrooted tree shows sequences from Viet Nam viruses compared with World Health Organization (WHO) reference strains for each genotype. RVi, rubella virus sequence from isolates.

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:

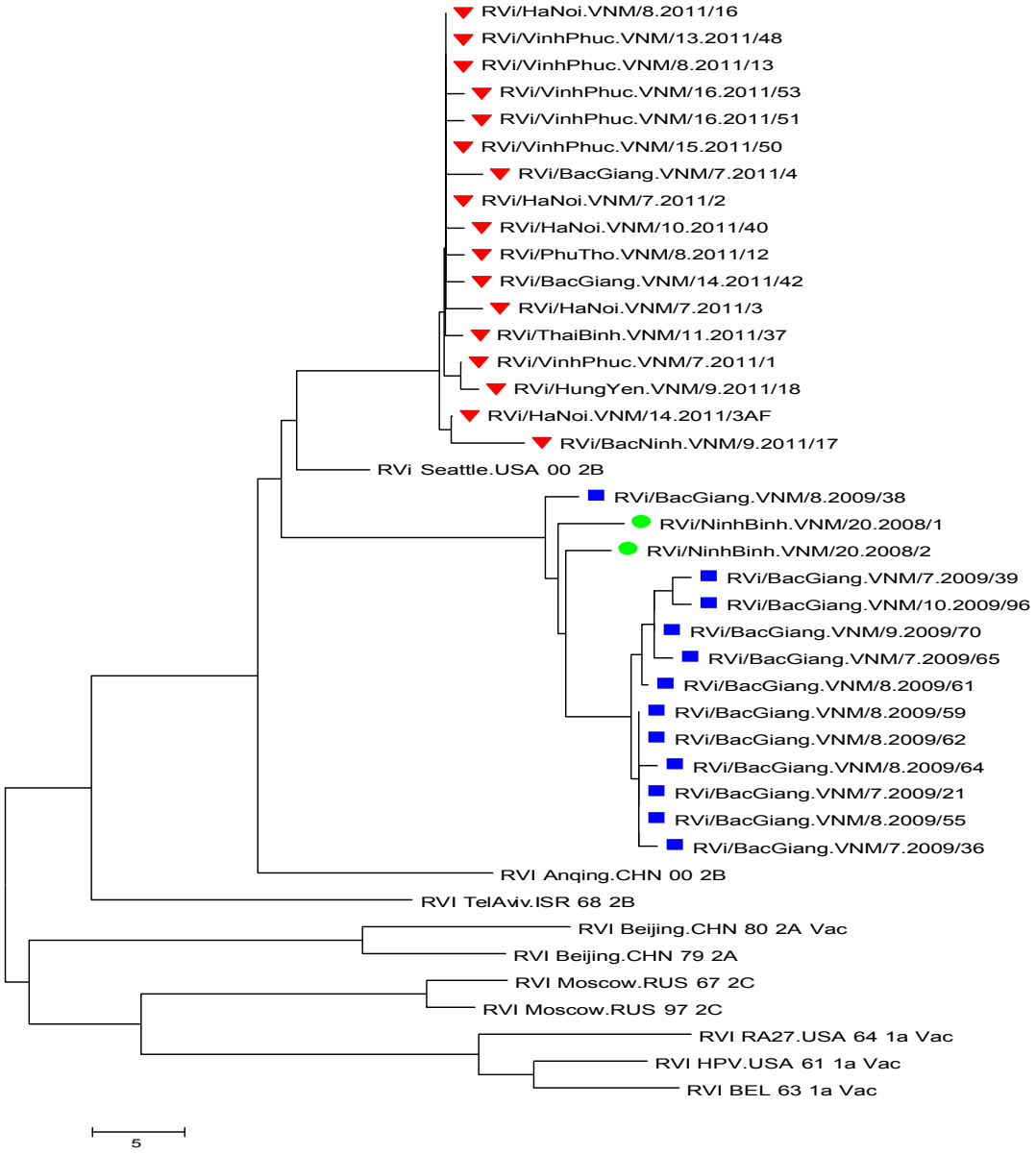

Figure 6. Phylogenetic analysis of the sequences of the E1 gene (739nt) of the strains of rubella virus from 2008-2011 and 3 reference strains for 2B .
Rvi, rubella virus sequence from isolates.
Sequences from RV isolated in 2008 are indicated by green.
Sequences from RV isolated in 2009 are indicated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

varied from 93% to 97% during 1993-2009, excluding 2007, when reported first-dose measles vaccine coverage was 83% because of a nationwide stock out. Measles vaccine first-dose is administered at 9 months of age, the second dose of measles vaccine was introduced in 2006 for children entering primary school and is administered at 6-7 years of age. Rubella vaccine was not introduced into the routine immunization.

Molecular epidemiologic data, when analyzed in conjunction with standard epidemiologic data, can help document viral transmission pathways, identify whether a virus is endemic or imported, and aid in case classification, thus enhancing control and elimination programs. Genetic analysis results showed that the H1 genotype virus was the predominant endemic measles virus in the North VietNam in 2006-2012. H1 genotype measles was also detected epidemic in Korea, China and in the centre of VietNam in 2000. In China, some studies showed that genotype H1 is the endemic genotype circulating in at least 16 years.

2B genotype of RV was circulating in some provinces in the North Viet Nam in 2008 - 2012. This genotype had a wide geographic distribution and were frequently found and were reported from 2 Middle Eastern countries, 5 European countries, 4 Southeast Asian, 4 South and Central American countries, 3 African countries and 2 Western pacific countries.

8.11. Reference

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8.12. 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) and WHO Vietnam country office;

Research 9

6.13 Title: Laboratory diagnosis of enteroviruses from cases with Hand, Foot, and Mouth Disease in 2011-2012 in Vietnam North

6.14 Name of researcher: Nguyen Thi Hien Thanh
Tran Nhu Duong

6.15 Specific objectives:

1. Identification of causative agents and epidemiological characteristics of HFMD
2. Identification of molecular characteristics of EV71,

Virological Investigation of Hand, Foot, and Mouth Disease, Northern Vietnam, 2011

6.16 Abstract

603 / 912 clinical samples from patients with hand, foot and mouth disease in 2011 collected from Northern provinces of Vietnam are positive with enteroviruses including 275 positive samples with HEV71 (45,6%) and 328 positive samples with other enteroviruses (54,4%). As the result of 328 sequenced PCR products, 177 samples were identified as Coxsackievirus A6 (53,9%), 102 as Coxsackie A16 (31,3%), 4,6% as other CoxsackieA viruses including type 3, 10, 12 and 13; 0,6% as Coxsackievirus type B; 4,3% as echo viruses; and 0,9% as Polio-Sabin virus and enteroviruse typ 96, 15 samples were identified as Rhinovirus (4,6%). Phylogenetic analysis of 49 HEV71 strains showed 2 genogroups B and C with 3 subgenogroups B5, C4 and C5 that cocirculated in norththern Vietnam in 2011. However, viruses belonging to subgenogroup C4 predominated with 73.5% and after is 20.4 % as B5, only 6.1% as subgenogroup C5 that circulated during a long period before. Disease was mainly detected in children under 3 years of age

Keywords: Hand, Foot and Mouth Disease, enterovirus, human enterovirus 71, coxsackie virus A6, A16; coxsackie virusB5; Echo virus30

6.17 Introduction

Hand, foot, and mouth disease (HFMD) is a common febrile illness of early childhood, characterized by 3–4 days of fever and the development of a vesicular enanthem on the buccal mucosa, gums, and palate and a papulovesicular exanthem on the hands, feet, and buttocks (*1*). HFMD is caused by acute enterovirus infections, particularly by viruses belonging to the human enterovirus A (HEVA) species

The genus *Enterovirus* of the family *Picornaviridae* is divided into 9 species, 5 of which infect humans. These viruses include the prototype species poliovirus, as well as HEVA, HEVB, HEVC, and HEVD. Viruses belonging to the HEVA species include 11 serotypes of coxsackievirus A (CVA; serotypes 2–8, 10, 12, 14, and 16), and human enterovirus 71 (HEV71)

Since the discovery of HEV71 in 1969 , numerous outbreaks of this infection have occurred throughout the world, the prevalence of HEV71 infection in the Asia-Pacific region has greatly increased since 1997, concurrent with an increase in the prevalence of HFMD, HFMD outbreaks have been recorded in Japan, Malaysia, Singapore, South Korea, the People's Republic of China, and Australia, The most extensive epidemic of HEV71 occurred in Taiwan in 1998, with ≈1,3 × 10^5 cases of HFMD, 405 cases related to severe neurologic syndrome , and 78 deaths, The deaths were due primarily to the development of brainstem encephalitis and neurogenic pulmonary edema

In 2003, we isolated HEV71/C4 from 01 patient with HFMD and have relation to severe neurologic syndrome, In 2008, a HFMD outbreak happened in the north of Vietnam and we isolated HEV71/C5 from 7 patients in this outbreak, Although laboratory surveillance has been shown to provide adequate warning of impending outbreaks of enteroviruses associated HFMD, laboratory surveillance for enteroviruses from patient with HFMD has not yet been established

A reverse transcription-seminested PCR (RT-snPCR) assay was developed for the detection RNA of EVs and EV71 from clinical specimens. The VP1 RT-snPCR assay was slightly more sensitive, and was used to identify EVs in clinical specimens. A product of the

expected size was successfully amplified and sequenced from clinical samples, The VP1 sequences derived from the RT-snPCR products allow rapid phylogenetic and molecular epidemiologic analysis of strains circulating during the EV season and comparison with EV sequences from past seasons or from different locations around the world,.

6.18 Materials and Methods

6.18.1 Specimen Collection

A total of 922 specimens were collected from the children with HFMD. Each child had at least 1 specimen collected from vesicle fluid, throat swab, or stool. 20% of specimens in PBS (+) were treateded with chloroform (1:10 in phosphate-buffered saline) before use for virus isolation in cell culture and molecular biology

Table1, Primer used for research

AN32	5-GTYTGCCA	cDNA	3009-3002	
AN33	5-GAYTGCCA		3009-3002	
AN34	5-CCRTCRTA		3111-3104	
AN35	5-RCTYTGCCA		3009-3002	
SO224-F	5-GCIATGYTIGGIACICAYRT	PCR1	2207-2226	762
SO222R	5-CICCIGGIGGIAYRWACAT		2969-2951	
AN89F	*5-CCAGCACTGACAGCA*GYNGARAYNGG	*PCR2/ EVs*	2602-2627	375
AN88R	*5-TACTGGACCACCTGG*NGGNAYRWACAT		2977-2951	
MAS01S	5'- ATAATAGCA(C/T)T(A/G)GCGGCAGCCCA -3')	*PCR2/ EV71*	2352-2375	376
MAS02A	5' – AGAGGGAG(A/G)TCTATCTC(C/T)CC -3')		2709-2728	

RNA extraction, Stool suspensions were prepared by adding 5 ml of phosphate-buffered saline (+), 1 g of glass beads (Corning Inc,, Corning, NY), and 0,5 ml of chloroform to 1 g of stool sample, shaking the mixture vigorously for 20 min in a mechanical shaker, and centrifuging at 1,500 _ *g* for 20 min at 4°C (33), the supernatant was transferred to a fresh tube, (10% stool suspensions), 140 _l of the specimen extract was combined with an equal volume of Vertrel XF (Miller-Stephenson Chemical Co,, Danbury, CT), shaken vigorously, and then centrifuged at 13,000 _ *g* for 1 min at room temperature, The aqueous phase was transferred to a fresh tube, Other specimen types (including blister fluid; throat swab samples) were processed without pretreatment, Twenty micrograms of proteinase K (Roche Applied Science, Indianapolis, IN) was added to 140 _l of each liquid specimen or fecal extract, and the mixture was then incubated for 30 min at 37°C, Nucleic acid was extracted from the digested specimen with a QIAamp Viral RNA mini kit (QIAGEN, Inc,, Valencia, CA), which was used according to the manufacturer's instructions, The eluted RNAs were passively dried in a benchtop desiccator under vacuum, The dried RNA was resuspended in 16 _l of sterile nuclease-free water and stored at _20°C until use,

RT-snPCR and sequencing.

- Synthesis of cDNA was carried out in a 5 µl reaction mixture containing 4 µl of RNA and 1 pmol each cDNA primer (primers AN32, AN33, AN34, and AN35; Table 1), heat mix in a 70^0C hear block for 5 min and immediately chill in ice water for at least 5 min.

In a 20 µl reaction mixture containing 05 µl RNA reaction mixture, 100 µM each deoxynucleoside triphosphate (dNTP; Invitrogen), 4 µl of 5 x reaction buffer (GoStript, Promega), 1.5 µl MgCl2, 20 U of RNasin (Promega Corp,, Madison, WI), and 100 U of GoScript reverse transcriptase (Rocke) , incubation at 25°C for 05 min, 42°C for 60 min, and 70°C for 15 min

- Synthesis of PCR1: Following incubation, the entire 05 µl RT reaction mixture was then used in the first PCR (final volume, 25 µl) (PCR1), consisting of 5 µl of 10 x PCR buffer (Roche Applied Science), 200 µM each dNTP, 50 pmol each of primers 224 and 222 (Table 1), and 2,5 U of *Taq* DNA polymerase (Roche Applied Science), with 40 cycles of amplification (95°C for 30 s, 42°C for 30 s, 60°C for 45 s),

One microliter of the first PCR was added to a second PCR (PCR2/EV71) for seminested amplification, PCR2/EV71 contained 40 pmol each of primers MAS01S and

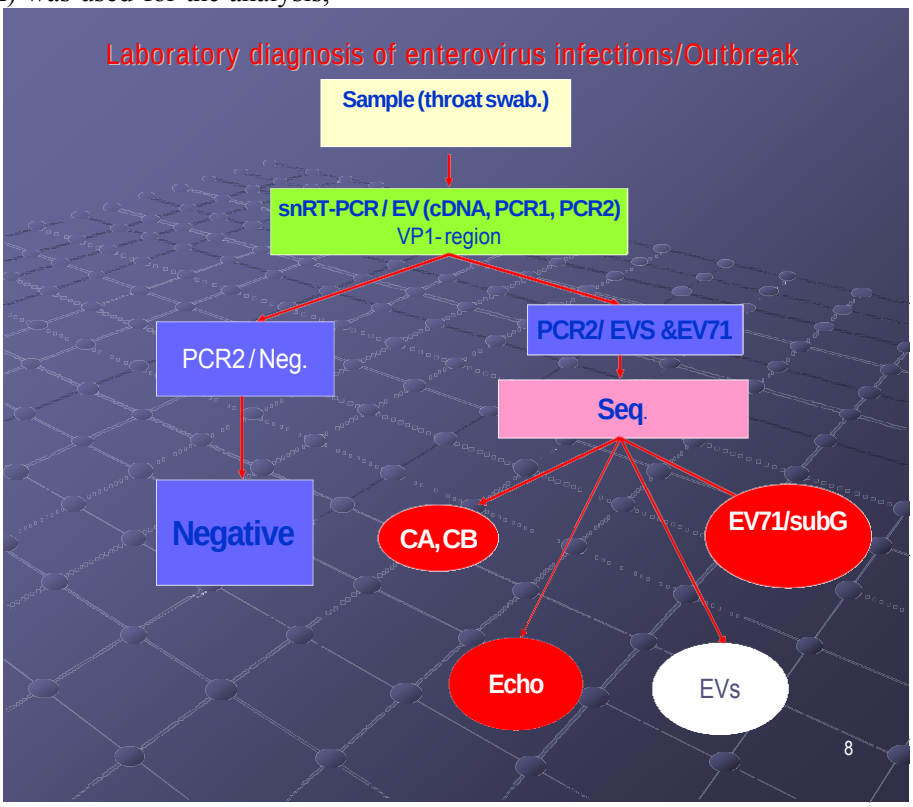
MAS02A (Table 1), 200 µM each dNTP, 5 µl of 10 x FastStart *Taq* buffer (Roche Applied Science), and 2,5 U of FastStart *Taq* DNA polymerase (Roche Applied Science) in a final volume of 50 µl, The FastStart *Taq* polymerase was activated by incubation at 95°C for 6 min prior to 40 amplification cycles of 95°C for 30 s, 60°C for 20 s, and 72°C for 15 s, The reaction products were separated and visualized on 2% agarose gels containing 0,5 µg ethidium bromide per ml

One microliter of the first PCR was added to a second PCR (PCR2/EV) for seminested amplification, PCR2 contained 40 pmol each of primers AN89 and AN88 (Table 1), 200 µM each dNTP, 5 µl of 10 x FastStart *Taq* buffer (Roche Applied Science), and 2,5 U of FastStart *Taq* DNA polymerase (Roche Applied Science) in a final volume of 50 µl, The FastStart *Taq* polymerase was activated by incubation at 95°C for 6 min prior to 40 amplification cycles of 95°C for 30 s, 60°C for 20 s, and 72°C for 15 s, The reaction products were separated and visualized on 1,2% agarose gels containing 0,5 µg ethidium bromide per ml and were purified from the gel by using a QIAquick gel extraction kit (QIAGEN), Slight variations in the sizes of the PCR products (350 to 400 bp) were observed due to VP1 gene length differences in the different serotypes, as described previously (12–14, 19), The resulting DNA templates were sequenced with a BigDye Terminator v1,1 ready reaction cycle sequencing kit on an ABI Prism 3100 automated sequencer (both from Applied Biosystems, Foster City, CA) by using primers AN89 and AN88 (Table 1),

Sequence analysis: The amplicon sequences were compared with the VP1 sequences of EV reference strains, including at least one representative of each recognized serotype, by script-driven sequential pair wise comparison with the program Gap (Wisconsin Sequence Analysis Package, version 10,2; Accelrys, Inc,, San Diego, CA), as described previously (15, 18, 19), In cases where the result was not unequivocal (highest score less than 75% or second-highest score greater than 70%), the deduced amino acid sequences were compared by a similar method,

6.18.2 Statistical Methods

Differences between proportions were tested by using the χ^2 test with Yates correction or Fisher exact test, Epi Info version 6 (Centers for Disease Control and Prevention, Atlanta, GA, USA) was used for the analysis,



Flow chart for detection of all enterovirus serotypes from clinical samples

6.19 RESULTS

6.19.1 Enteroviruses were protected by snRT-PCR and Seq.

Table1. Result of Enteroviruses anf EV71 by snRT-PCR in 2011

N report	N collected samples	% tested	N (+)	% (+)	(+) EV71		(+) Enterovirus	
					N(+)	%(+)	N(+)	%(+)
20.529	912	4.4	603	63.5	275	45.6	328	54.4

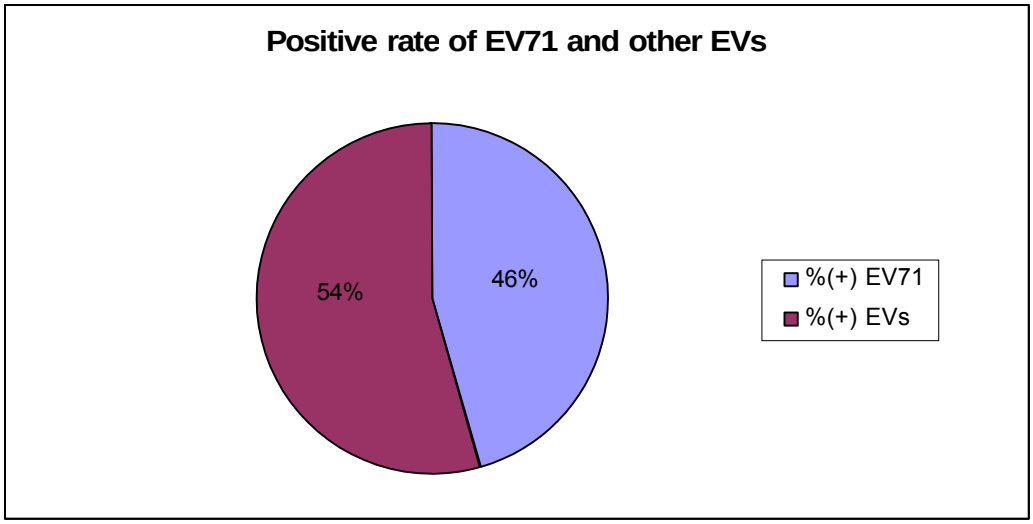


Figure 1. HEV-71 and EVs was protected by snRT-PCR

In 2011, 603 of 912 HFMD cases (63.5%) were found positive for HEV by using enterovirus general primers, and snRT-PCR method. HEV-71 and EVs occupied 46% (275) and 54% (328) respectively. (Figure 1).

Table2. Result of enterovirus serotypes by sequencing in 2011

N enterovirus by snRT-PCR	Result of Enterovirus serotypes by seq.						
	(+) Coxsackievirus - A			(+) CB	(+) Echo	(+) Other EV	Rhinovirus
	CAV- 6	CAV-16	Other CAV				
328	177 53,9%	102 31,3%	15 4,6%	2 0,6%	14 4,3%	3 (EV96 & Polio) 0,9%	15
	89,6%			5,8%			4,6%

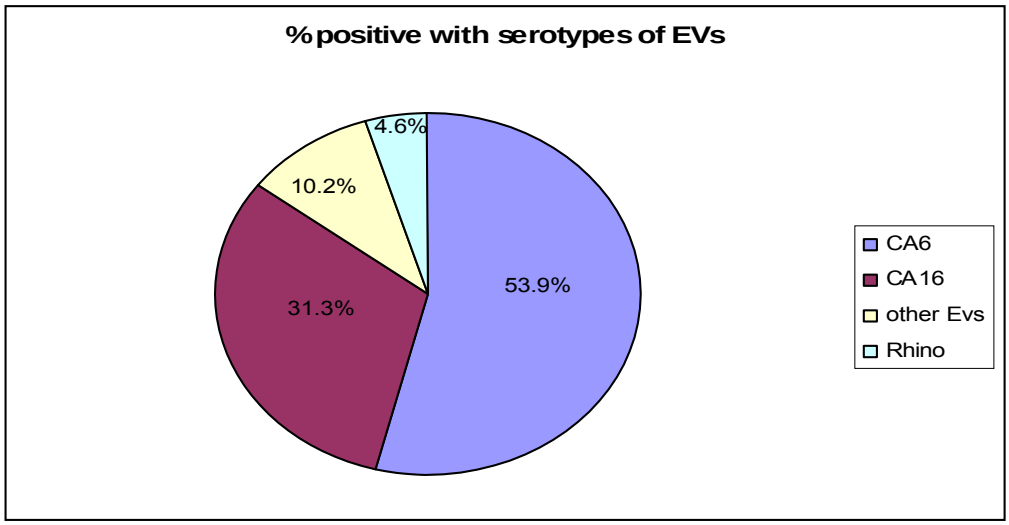


Figure 2. Enterovirus Serotypes was confirmed by Sequencing

328 of other EVs PCR-2 products were sequenced with primer pairs at position of primer F 2602-2627 or primer R 2977-2951 (375nt). The result showed with 53,9% of CoxsackieA 6; 31,3% of CoxsackieA16. The other entroviruses containing 10.2% with CV-A3(1), A10(4), A12 (8), và A13 (2); CV-B3, B4 (2);14 echovirus type 30 and 2 Polio, 1 EV96 were also detect (Figure 2)

Table 3. Enterovirus type 71's subgenogroups

TT of PCR produts/EV71	TT of EV71 PCR products sequenced	C4	C5	B5
275	49	36	3	10
% of each EV71 subgenogroup		73.5	6.1	20.4

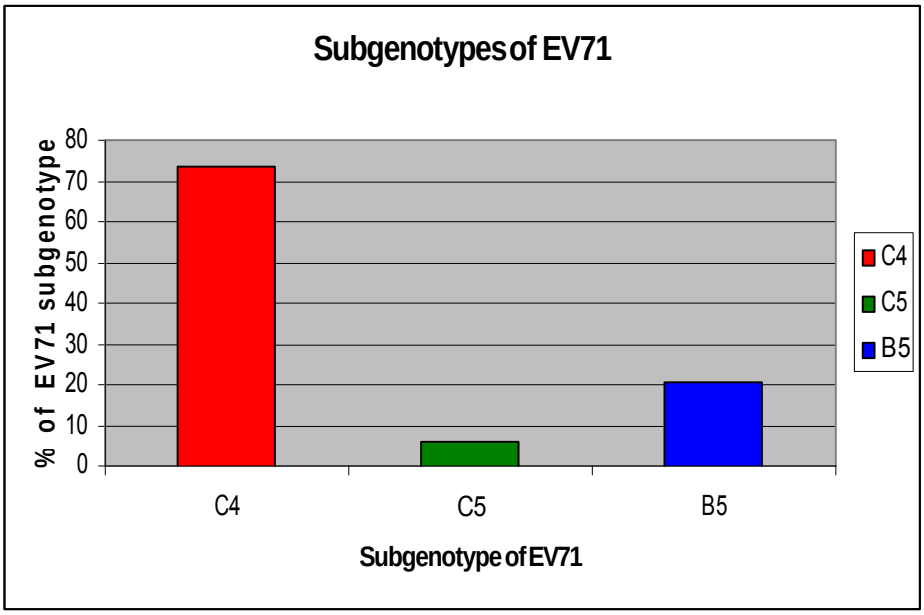


Figure 3. Enterovirus type 71's subgenogroups

49 PCR products of EV71 were amplified and sequenced. Result showed 73.5% as subgenotype C4, 6.1% as subgenotype C5 and 20.4 % as subgenotype B5

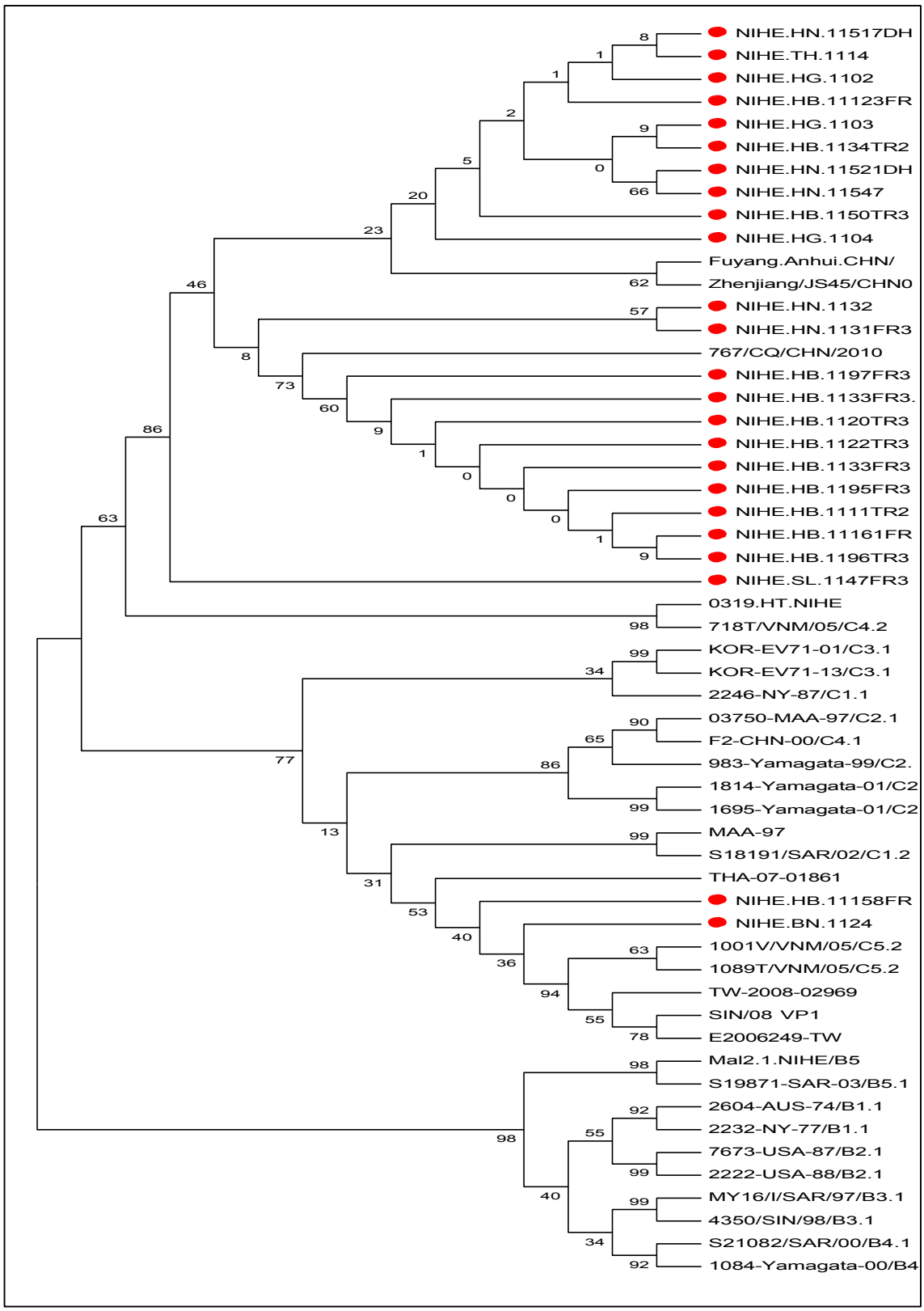


Figure 4. Phylogenetic relationships of human enterovirus 71 (HEV71) strains belonging to genogroup C

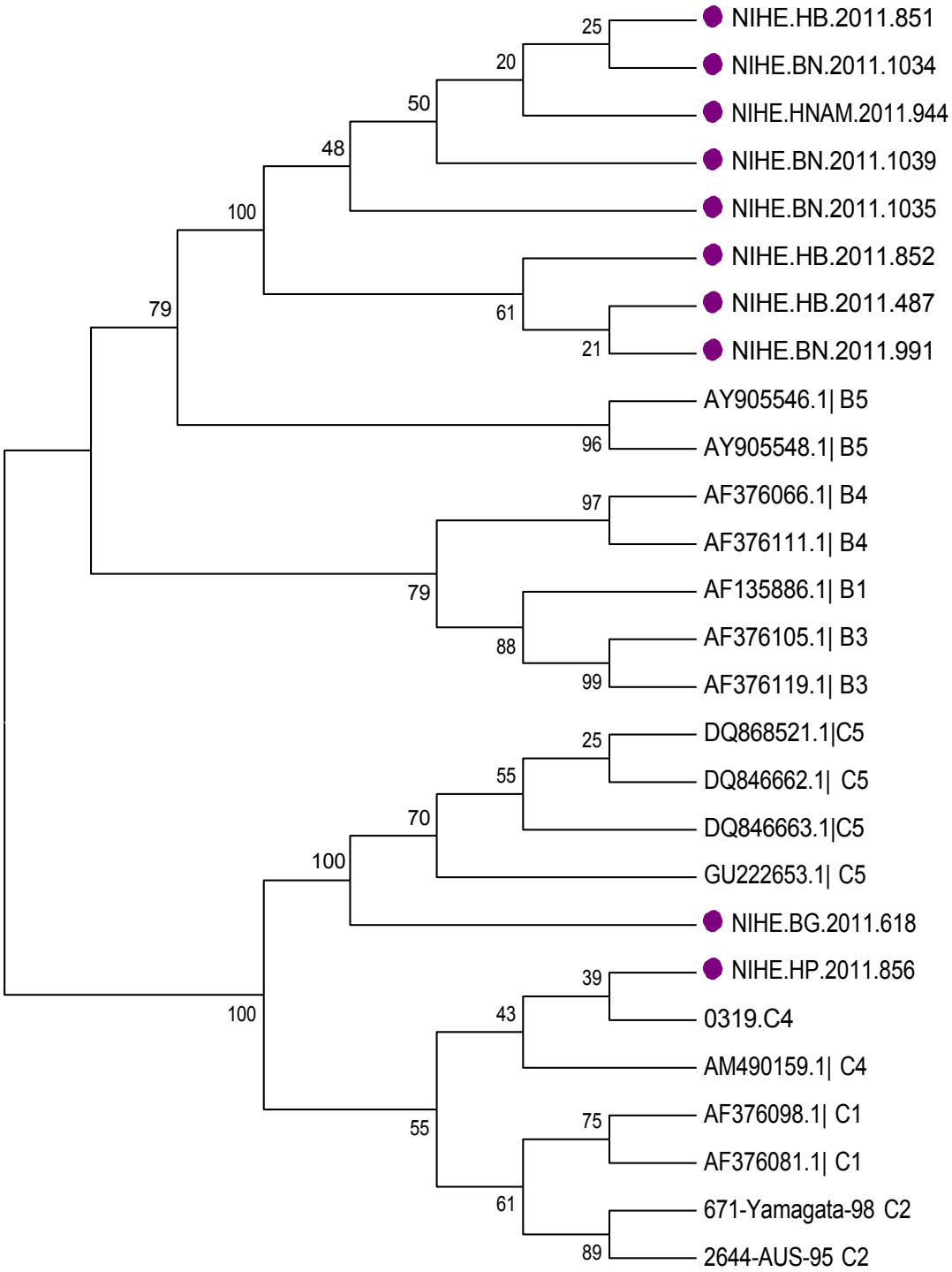


Figure 5. Phylogenetic relationships of human enterovirus 71 (HEV71) strains belonging to genogroup B and C

49 HEV-71 PCR products from 21 isolates and 28 clinical samples were sequenced. Result showed 79.6 % belonging to genogroup C (Figure 3,4) with 73.5% of subgenogroup C4 predominantly responsible for almost all HEV-71 infections in 2011 in the north of VN and 6.1% of subgenogroup C5, and 20.4% belonging to genogroupB, subgenogroup B5 (Figure4). However, the analysis of recent and previous HEV71 isolates in the Western Pacific Region showed that several subgenogroups, B1, B2, B3, B4, C1, C2, C3 and C4 were cocirculating in Australia, Malaysia, Singapore, Taiwan and Japan respectively.

Table 4. Ages distribution of 603 cases with HFMD confirmed as EV71 and other enteroviruses

Age	N tested	N(+)	N (+) VRĐR	(+) EV71		(+) VRĐR khác	
				N(+)	% (+)	N(+)	%(+)
<1	97	62	10.3%	22	8.0%	40	12.8%
1	329	232	38.5%	97	35.3%	135	43.1%
2	261	183	30.3%	87	31.6%	96	30.7%
3	103	57	9.5%	33	12.0%	24	7.7%
4	37	22	3.6%	13	4.7%	9	2.9%
5	30	15	2.5%	7	2.5%	8	2.6%
6 -30	39	20	3.3%	12	4.4%	8	2.6%
No infn	16	12	2.0%	4	1.5%	8	2.6%
TT	912	603	100%	275	100%	313	100%

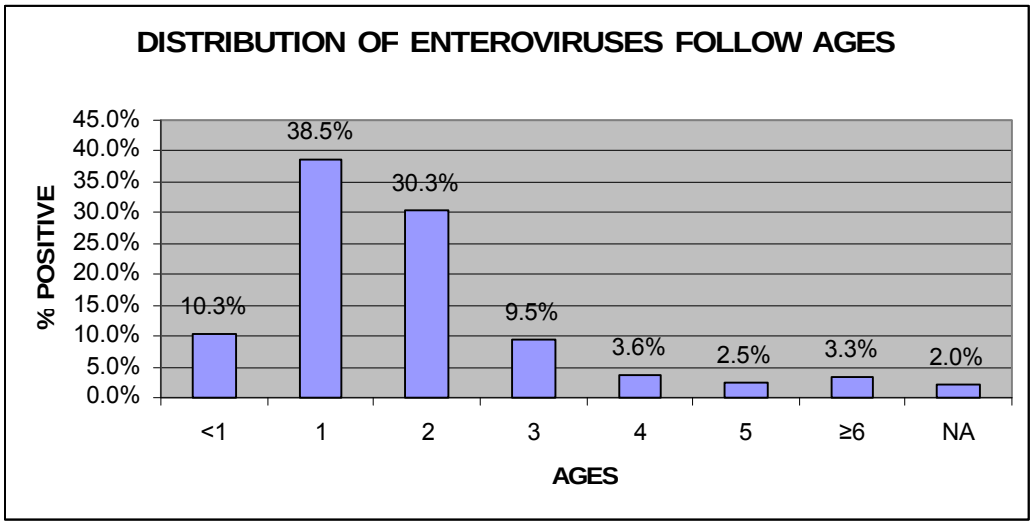


Figure 6, Ages distribution of 603 cases with HFMD confirmed as enteroviruses

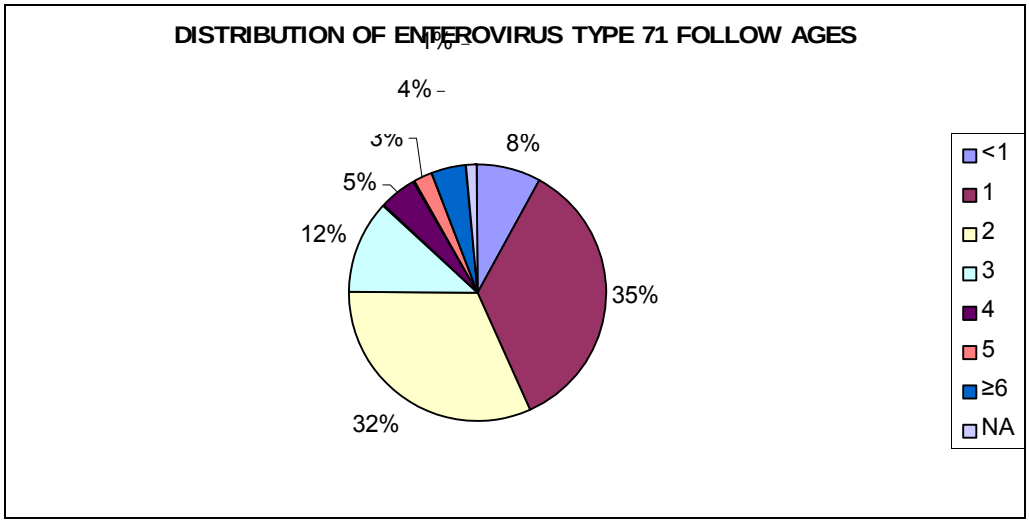


Figure 7, Ages distribution of 275 cases with HFMD confirmed as enterovirus TYPE 71

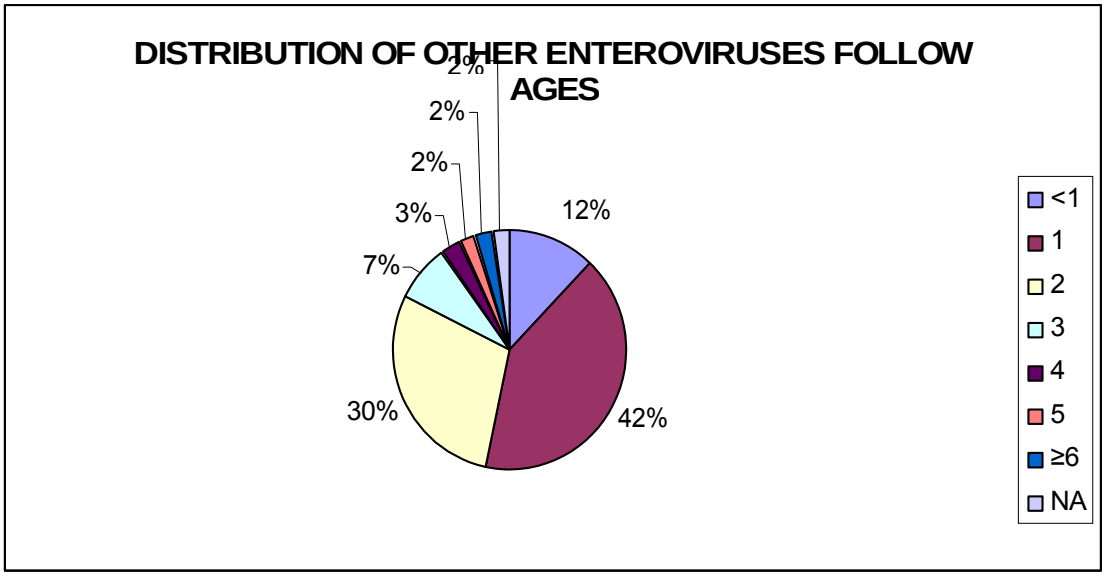


Figure 8, Ages distribution of 328 cases with HFMD confirmed as other enteroviruses

Table 4 showed the results of HFMD following age group, the disease is highly concentrated in children within 3 years, there is no difference of the incidence of diseases caused by enterovirus type 71 (46.7%) and other enterovirus (53.3%) in each age group.

Table 5. Geographic distribution of hand, foot, and mouth disease cases

IT	Provinces	N-report	N-tested	(+) Enteoviruses	% (+) EVs	(+) EV71	% (+)	(+) other EVs	% (+)
1	LẠNG SƠN	662	2	1	0.2%	0	0,0	1	100,0
2	HƯNG YÊN	154	3	2	0.3%	0	0,0	2	100,0
3	T, NGUYÊN	224	6	2	0.3%	0	0,0	2	100,0
4	LAI CHÂU	50	6	3	0.5%	2	66,7	1	33,3
5	NGHỆ AN	561	10	4	0.7%	1	25,0	3	75,0
6	SƠN LA	174	6	5	0.9%	5	100,0	0	0,0
7	ĐIỆN BIÊN	28	10	6	1.0%	5	83,3	1	16,7
8	THÁI BÌNH	814	12	6	1.0%	1	16,7	5	83,3
9	HÀ NAM	270	16	7	1.2%	2	28,6	5	71,4
10	CAO BẰNG	257	10	9	1.5%	1	11,1	8	88,9
11	HÀ TĨNH	118	13	9	1.5%	1	11,1	8	88,9
12	LÀO CAI	88	10	9	1.5%	4	44,4	5	55,6
13	YÊN BÁI	495	11	9	1.5%	2	22,2	7	77,8
14	NINH BÌNH	1,028	33	15	2.6%	1	6,7	14	93,3
15	QUẢNG NINH	473	25	16	2.7%	2	12,5	14	87,5
16	HÀ GIANG	285	33	20	3.4%	10	50,0	10	50,0
17	NAM ĐỊNH	133	25	21	3.6%	9	42,9	12	57,1
18	BẮC CẠN	380	31	22	3.7%	9	40,9	13	59,1
19	VĨNH PHÚC	872	38	23	3.9%	2	8,7	21	91,3
20	HẢI DƯƠNG	536	45	24	4.1%	1	4,2	23	95,8

21	BẮC NINH	158	42	25	4.3%	11	44,0	14	56,0
22	T, QUANG	561	24	26	4.4%	15	57,7	11	42,3
23	PHÚ THỌ	967	77	31	5.3%	1	3,2	30	96,8
24	THANH HÓA	3,744	49	33	5.6%	25	75,8	8	24,2
25	BẮC GIANG	508	60	46	7.8%	8	17,4	38	82,6
26	HẢI PHÒNG	3,046	80	63	10.7%	56	88,9	7	11,1
27	HÀ NỘI	1,579	116	70	11.9%	32	45,7	38	54,3
28	HÒA BÌNH	2,364	119	81	13.8%	69	85,2	12	14,8
	Tổng	20,529	912	588		275	46,8	313	53,2

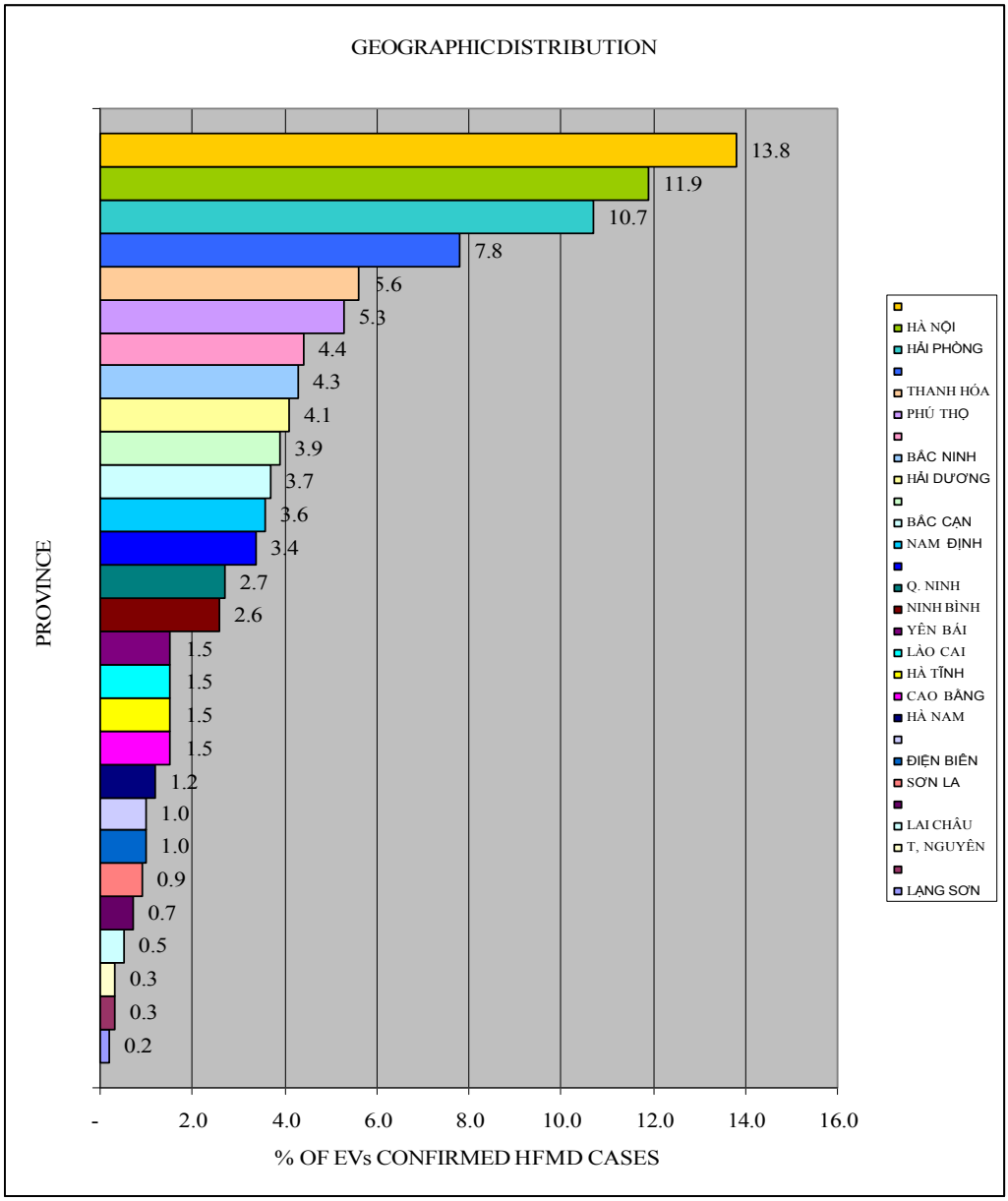


Figure 9. Geographic distribution of hand, foot, and mouth disease cases

The results showed 28/28 Northern provinces had HFMD cases. However, the HFMD cases in delta area was higher than mountain area; 24/28 provinces with the co-circulation of EV71 and other EVs. One only province (Son La province) only detected EV71 and three provinces (Hung Yen, Lang Son and Thai Nguyen province) only detected enterovirues

Table 6. Geographic distribution of hand, foot, and mouth disease cases by EV71, CAV-6 and CAV-16

PROVINCE	% (+)/ EV71	% (+)/ CA16	% (+)/ CA6	% (+)/ Other EVs
BẮC CẠN	40.9%	9.1%	50.0%	0.0%
BẮC GIANG	18.2%	22.7%	47.7%	9.1%
BẮC NINH	50.0%	18.2%	22.7%	4.5%
CAO BẰNG	11.1%	88.9%	0.0%	0.0%
ĐIỆN BIÊN	83.3%	16.7%	0.0%	0.0%
HÀ GIANG	55.6%	0.0%	38.9%	5.6%
HÀ NAM	28.6%	42.9%	28.6%	0.0%
HÀ NỘI	48.5%	20.6%	25.0%	2.9%
HÀ TĨNH	10.0%	0.0%	80.0%	0.0%
HẢI DƯƠNG	0.0%	56.5%	26.1%	17.4%
HẢI PHÒNG	100.0%	0.0%	0.0%	0.0%
HÒA BÌNH	93.3%	0.0%	4.0%	0.0%
HƯNG YÊN	0.0%	50.0%	50.0%	0.0%
LAI CHÂU	40.0%	20.0%	0.0%	0.0%
LẠNG SƠN	0.0%	100.0%	0.0%	0.0%
LÀO CAI	33.3%	50.0%	0.0%	16.7%
NAM ĐỊNH	42.9%	14.3%	42.9%	0.0%
NGHE AN	20.0%	20.0%	20.0%	0.0%
NINH BÌNH	7.7%	7.7%	69.2%	7.7%
PHÚ THỌ	3.3%	23.3%	46.7%	26.7%
QUẢNG NINH	13.3%	6.7%	73.3%	6.7%
SƠN LA	100.0%	0.0%	0.0%	0.0%
T. NGUYÊN	0.0%	0.0%	100.0%	0.0%
T. QUANG	47.6%	0.0%	38.1%	9.5%
THÁI BÌNH	14.3%	0.0%	85.7%	0.0%
THANH HÓA	64.5%	12.9%	9.7%	3.2%
VĨNH PHÚC	8.3%	79.2%	12.5%	0.0%
YÊN BÁI	33.3%	33.3%	33.3%	0.0%

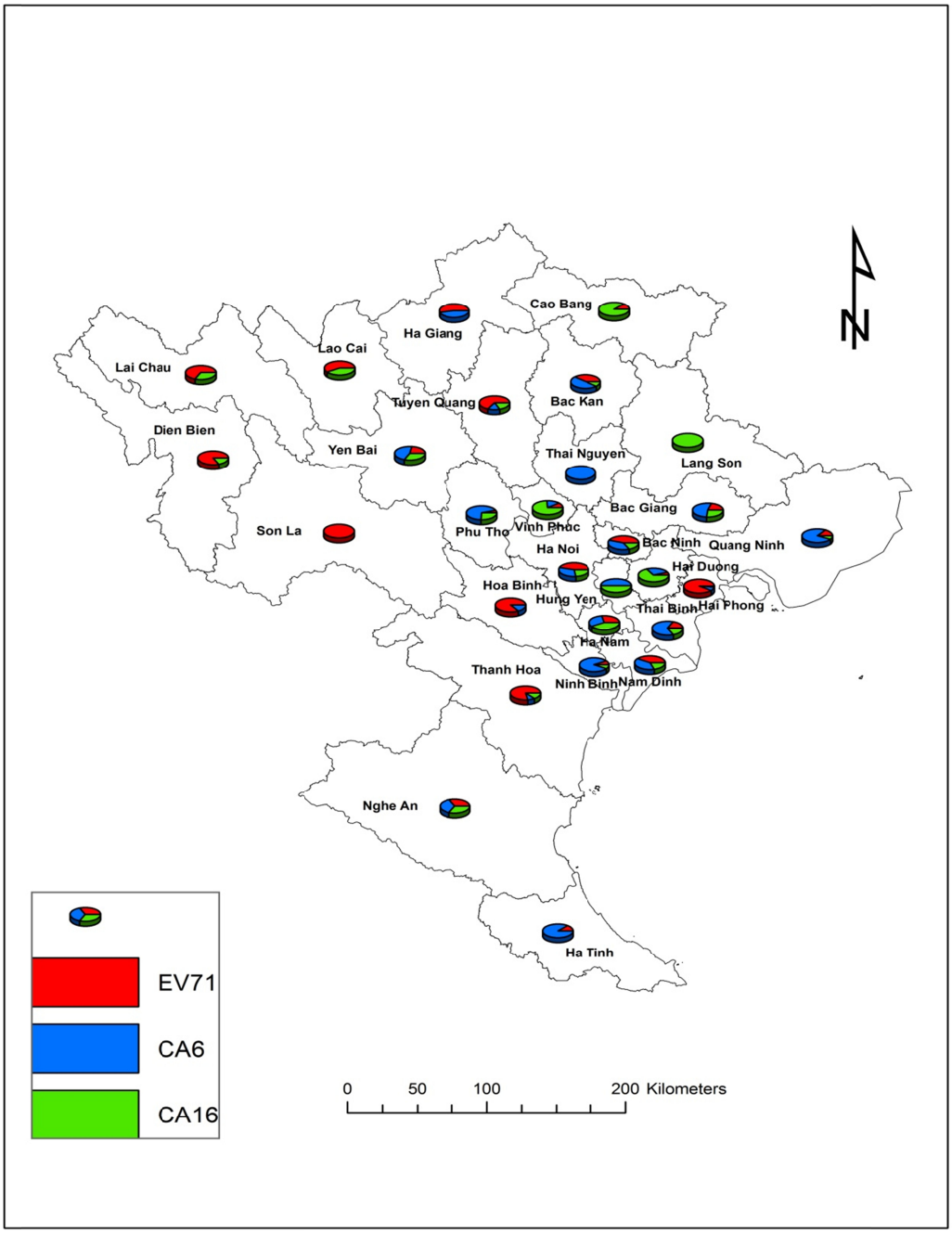


Figure 10. Geographic distribution of hand, foot, and mouth disease cases associated with infection of human enterovirus 71, coxsackievirus A6 and coxsackievirus A6 in Northern Vietnam, 2011.

After circulation of EV71 is CAV 6 and CAV 16. There are difference for circulating of serotypes of Coxsackievirus-A in the northern provinces, some provinces with only circulation of Coxsackievirus-A6 (Ha Tinh, Thai Nguyen, Ha Giang, Hoa Binh, Tuyen Quang), and some provinces have only Coxsackievirus A16 (Cao Bang, Dien Bien, Lai Chau, Lang Son and Lao Cai), the remaining provinces have co-circulation of both Coxsackievirus-A6 and CAV16

Some provinces detected Coxsackievirus A3 (Bac Giang), Coxsackievirus A10 (Bac Giang, Lao Cai, Hai Duong, Phu Tho), Coxsackievirus A12 (Bac Giang, Phu Tho, Hanoi, Hai Duong, Quang Ninh), Coxsackievirus A13 (Tuyen Quang), echovirus type 30 in Phu Tho and

Ninh Binh, Ha Giang, Bac Ninh; especially with appearance of enterovirus type 96 in Tuyen Quang province.

Table 7. Geographic distribution of hand, foot, and mouth disease cases by EV71 subgenogroup

IT	PROVINCE	N - EV71	C4	C5	B5
1	BẮC GIANG	2		1	1
2	BẮC NINH	5	1		4
3	HÀ GIANG	4	4		
4	HÀ NAM	1			1
5	HÀ NỘI	6	6		
6	HẢI PHÒNG	6	6		
7	HOÀ BÌNH	17	14		3
8	SƠN LA	1	1		
9	THANH HOÁ	5	4	1	
	TT	47	36	2	9

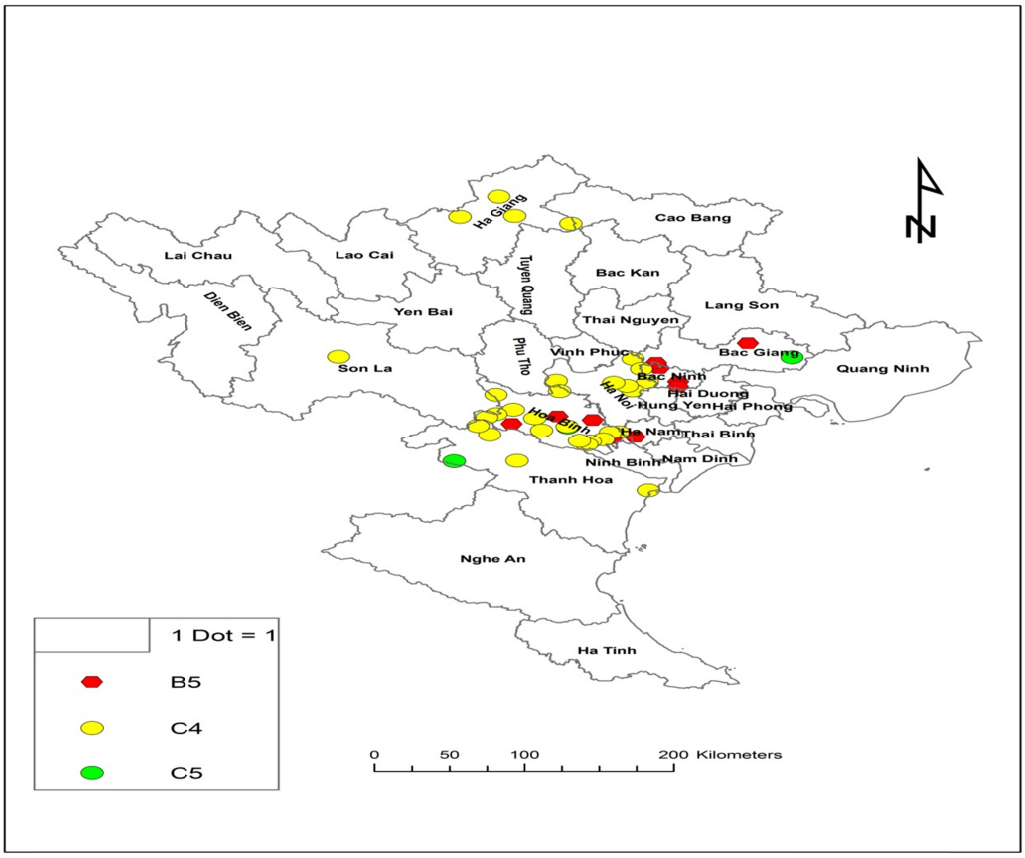


Figure 11. Geographic distribution of hand, foot, and mouth disease cases associated with infection of subgenogroup/HEV-71, in Northern Vietnam, 2011.

46 EV71 from 9 provinces in the north of Viet Nam were sequenced and analyzed. Results showed, they belonged to 2 genogroups with 3 subgenogroups B5, C4 and C5. Bac Ninh and Hoa Binh province had co-circulation of 2 genogroups B and C with subgenogroup B5 and C4; Bac Giang province had also co-circulation of 2 genogroups B and C with subgenogroup B5 and C5; Thanh Hoa province had circulation of genogroup C with 2 subgenogroups C4 and C5

6.20 DISCUSTION

6.20.1 Epidemiology of HFMD

HFMD was identified in northern Vietnam in 2011; HEV71, CVA16 and CA6 were also identified throughout the year, EV71 was the predominant virus during this time, accounting for 46% (275 cases) of HFMD compared to 54% (328 cases) for all other enteroviruses (table 1). After EV71 were CA6 and CA16, they were also the predominant viruses during this time, accounting for 53.9 % of HFMD compared to 31.3% for CVA6 and CA16 respectively (table 2).

Enteroviruses have circulated and caused HFMD outbreak in 2011 at all 28 northern provinces (table 5 and figgue 9)

6.20.2 Molecular Epidemiology of HEV71

Phylogenetic analysis based on nucleotide sequence alignment of 18 representative strains with the complete VP1 gene and 28 PCR products from clinical samples of HEV71 at 9 provinces of northern Vietnam. Result showed 2 genogroups that belonged to genogroup C with 2 subgenogroups C4, C5 and genogroup B with subgenogroup B5. Predominant EV71 strain in 2011 identified as subgenotype C4 during the HFMD outbreak of year and after subgenotype C4 was subgenogroup B5. Subgenogroup C5 was sporadic

Genogroup C with subgenogroup C4 and C5 have emerged recently in Southeast Asia, Viruses belonging to subgenogroup C4 were first identified in the People's Republic of China in 1998 and again in 2000 before their identification in northern Vietnam in 2003 (only one case), Furthermore, a new subgenogroup C5, circulated in northern Vietnam throughout 2008-2010 but decreased in 2011 and subgenogroup C4 reappeared and replaced for subgenogroup C5, and a new genogroup (genoproup B) with subgenogroup B5 appeared and caused HFMD in 2011

With evidence of the ongoing evolution of new subgenogroup and appearance of new genogroup similar to that observed for genogroup B HEV71 strains in Southeast Asia . Furthermore, the year-round detection and circulation of multiple independent genetic lineages of HEV71 suggested that this virus have circulated endemically within the human population of northern Vietnam.