2.11. Reference

1. GARP-Việt Nam (2010). Phân tích thực trạng: Sử dụng kháng sinh và kháng kháng sinh ở Việt Nam.
2. 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.
3. Nordmann P, Cuzon G, Naas T. The real threat of *Klebsiella pneumoniae* carbapenemase-producing bacteria. *Lancet Infect Dis* 2009; 9: 228–36.
4. Yong D, Toleman MA, Giske CG, et al. 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* 2009; 53: 5046–54.

2.12. Acknowledgements

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 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 Objectives

a. To identify molecular characterization of *V. Cholerae* strains isolated from cholera outbreaks in Vietnam from 2007 to 2009 by using PFGE and MLVA methods
b. To investigate relationships between the *V. cholerae* O1 isolates from cholera outbreaks in Vietnam from 2007 to 2009
c. To compare molecular characterization of *V. cholerae* O1 isolated from patient and enviroment
d. 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: Molecular epidemiologic analysis of *V. cholerae* O1 isolates in Vietnam from 2007 to 2009 by Pulsed-Field Gel Electrophoresis

3.7 Summary

In this study, we analyzed 100 of *V. cholerae* isolates which were collected from patients and 4 the environmental isolates in northern Vietnam during period 2007 to 2009. Of the 100 isolates, 25 were collected in 2007, 37 were collected in 2008 and 38 were collected in 2009. All of the 98 isolates were identified as being of the *V. cholerae* O1 Ogawa serotype. Multiplex PCR were used to identify the specific genes of *V. cholerae,* positive for V.O1, *ctx A, ToxA* genes. The result of PFGE showed that all the 98 isolates during period 2007 to 2009 in Vietnam with very similar PFGE pattern (shared a coefficient of similarity at ≥ 93%) are clonally related or belong to a single clonally cluster, which includes isolates of human and environmental origin

3.8 Purposes

Cholera is anacute diarrhoea caused by *Vibrio cholerae* O1 and O139. Before 2005, only a few cases of cholera were reported in the Northern part of Vietnam. However, by the end of 2007, major outbreaks of cholera were occurring in this region (5). From 24 October 2007 to 25 June 2009, there were more than 7,000 cases with acute severe, watery diarrhoea in 22 cities and provinces in Northern Vietnam. In order to investigate the origin of strains in domestic cases, a total 100 strains of *V. cholerae* O1 isolated from patients and enviroment in period 2007 to 2009 were analysed by PFGE for genotyping. Our data should provide useful epidemiological baseline information with public-health implications, such as for epidemic tracing of indigenous strains and for identifying their genetic relationship with the strains that may emerge in the future (2,3,4,7).

3.9 Methods.

3.9.1 ***Culture method***

One hundred of *V. cholerae* O1 isolates from patients and environment (water samples, food, vegetable) collected from different provinces and different years (from 2007 to 2009) in Vietnam. To confirm the

V. cholerae O1 isolates by culture method. The *V. cholerae* isolates were tested by agglutination reaction with polyvalent O1 antiserum, and the strains that gave a positive agglutination were serotyped using monovalent Ogawa and Inaba antisera (Denka Seiken, Japan). All the isolates were of the Ogawa serotype. The *V. cholerae* O1 isolates were stored in LB broth (Sigma) containing 15% glycerol at -70 °C until use.

The strains were used as references: *V. cholerae* O1 - H218 and *V. cholerae* O139 - AI 4450 for positive controls and *Salmonella* Braenderup H9812 for molecular mass marker.

3.9.2 *Multiplex-PCR (Polymerase Chain Reaction)*

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 1□□□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.

3.9.3 *Pulsed-Field Gel Electrophoresis (PFGE)*

Pulsed-field gel electrophoresis was performed according to the standardized PulseNet PFGE protocol (6) . After embedded in the agarose blocks, bacteria were lysed in the agarose plugs and incubated overnight. The next day, the plugs were rinsed and sliced to 2.0-2.5 mm thickness. Slices of agarose plug were digested with 20 units of *Not*I restriction endonuclease (Promega, Madison, WI, USA). PFGE was carried out using a CHEF-DRIII system (Bio-Rad, Laboratories, Richmond, CA, USA) through 1.2% agarose with ramp times of 7-12 sec for 11.5 h, followed by 9-12 sec for 10.5 h for *Not*I. Electrophoresis was performed at 14°C at 6 volts/cm with a 120° reorienting angle. *Salmonella* Braenderup H9812 was used as the molecular mass marker. Gels were then stained with ethidium bromide (0.5 μg/mL) for 30 min, rinsed 3 times for a total of 30 min, and visualized by UV transillumination. Photographs were taken and filed.

The images of DNA band patterns were analyzed for cluster analysis using Molecular Fingerprinting Analyst (Bio-Rad, USA) sofware based on the Dice similarity coefficient and unweighted pair-group method with arithmetic averages.

Analysis of PFGE patterns

V. cholerae O1 chromosomal restriction PFGE patterns were classified according to Tenover *et al.* (9) and Arakawa *et al.* (1). When four or more DNA bands in the PFGE patterns were different from each other, we assigned them as different patterns by Arabic numerals, i.e. patterns 1 to 17. Patterns with less than a four-band difference were considered subtypes, i.e. 2a, 2b, 2c, 2d, 2e, 8a, 8b, 8c, 11a, 11b, 11c, and 11d. The DNA restriction PFGE patterns obtained were also saved as TIFF files for use with Bio-profil (Vilber Lourmat, Marne-La-Vallée, France). For the latter, normalization was done according to the molecular size standards of each gel, with one molecular weight standard being used for 3-4 samples. Construction of similarity matrices was carried out by comparison of Dice coefficients (3,8). The band-based Dice coefficient is based on a comparison of designated band positions by dividing the number of matching bands between patterns by the total number of bands, thereby emphasizing the matching bands. In all the cases, an un-weighted pair group matching band average (UPGMA) at a 1.3% tolerance window was used for clustering the pulsed-field gel electrophoresed (PF) patterns.

3.10 Results

3.10.1. PCR analysis

Multiplex PCR were used to identify the specific genes of *V. cholerae:* V.O1,V.O139, *ctx A, ToxA*. The data showed that the 96 of clinical isolates and 4 of environmental isolates tested were all positive for V.O1,*ctx A, ToxA* genes.

Year of isolation	Number of isolates	Presence of genes			
		ctxA	V.O1	V.O139	ToxA
2007	25	+	+	–	+
2008	37	+	+	–	+
2009	38	+	+	–	+
	Total: 100				

3.10.2. PFGE analysis

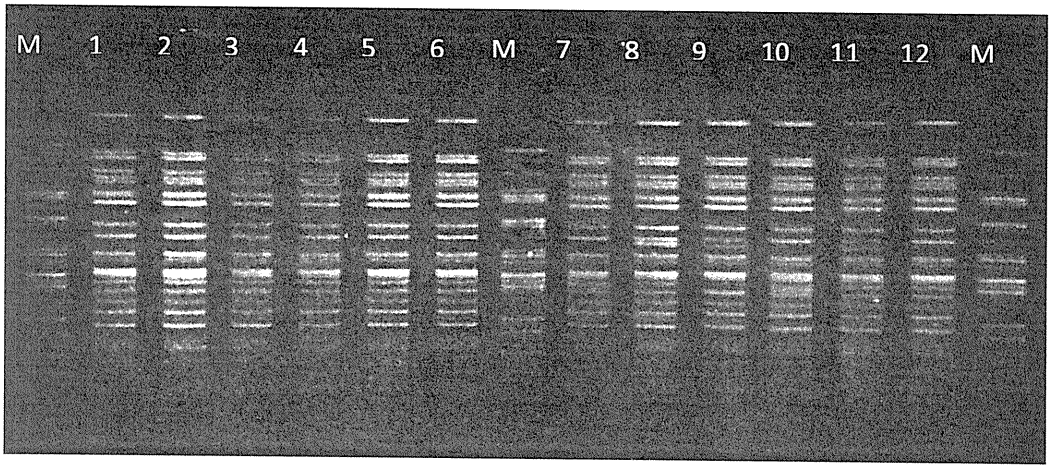


FIG.1. Representatives PFGE patterns of *V. cholerae* O1 strains cleaved with restriction *NotI*. Lanes 1-7 and 10, 12 (VC70-07, 75-07, 229-08, 310-08, 167-09, 7: 99-09, 316-07, 211-09) show representatives PFGE patterns of type. Lanes 8-9 and 11 (48-07, 51-07, 324-07)

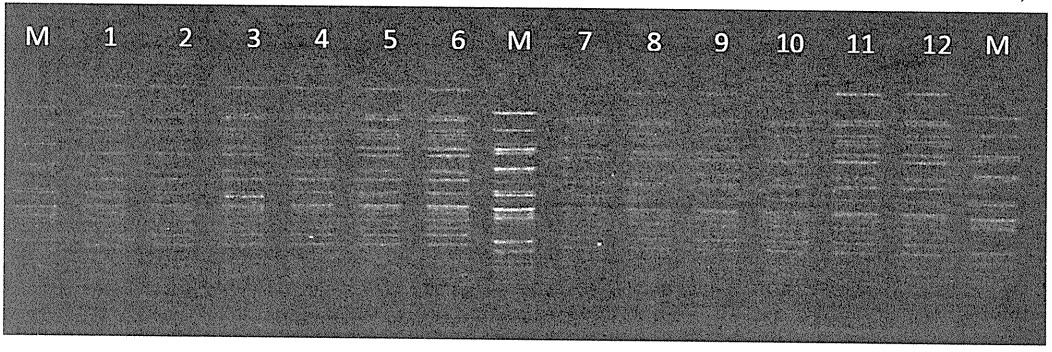
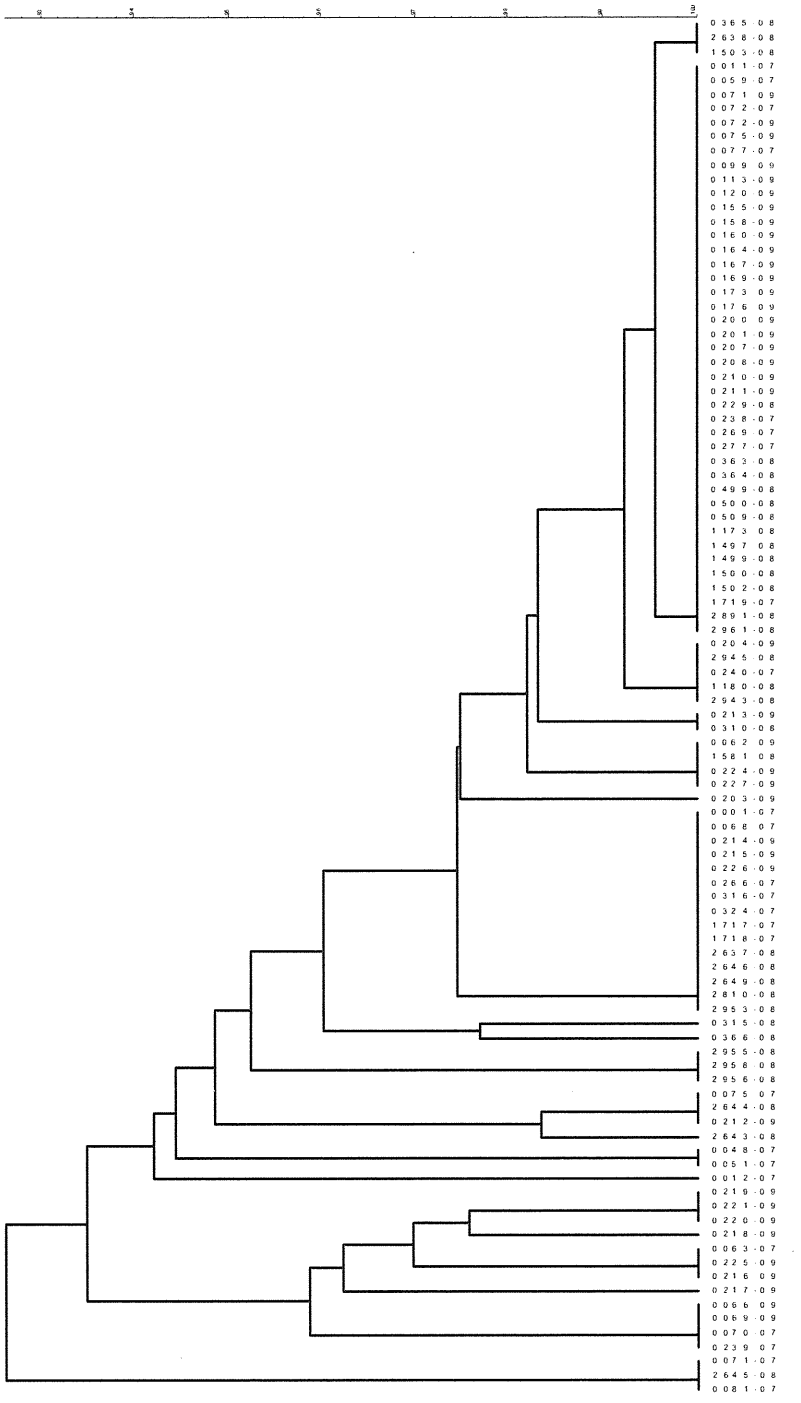


FIG.2. Comparison of Representatives PFGE patterns of *V. cholerae* O1 clinical strains and enviromental strains. Lanes 1-4: *V. cholerae* O1 enviromental strains (VCE11-07, 12-07, 81-07, 217-07).

Lanes 5-12: *V. cholerae* O1 clinical strains (VC0225-09, 0227-09, 59-07, 269-07, 363-08, 2691-08, 169-09, 213-09) show representatives PFGE another patterns of type.

Lane M: Molecular mass Marker: *Salmonella* Braenderup H9812 .

FIG.3. Dendrogramme and PFGE pattern related following the year of isolates



3.11 Discussion

One hundred of *V. cholerae* O1 isolates collected from different provinces during various times (in period 2007 to 2009) exhibited very similar, although not entirely identical, PFGE banding patterns which suggest that they are clonal. The numerical similarities between isolates, as defined by Dice coefficients, were in the ranges of 0.93-1.0 by using *Not*I. The high Dice coefficients represented the high similarities among chromosomal restriction fragment patterns. These isolates are epidemiological

related isolates. The banding pattern of clinical isolates in PFGE was similar from those of the environmental isolates.

The result of PFGE showed that all the ctxA positive strains of *V. cholerae* O1 with very similar PFGE pattern are clonally related or belong to a single clonally cluster, which includes isolates of human and environmental origin.

PFGE was found to be a useful method for investigating the source, transmission, and spread of cholera infection.

3.12 Publications

None

3.13 Reference

1. Arakawa E, Murase T, Matsushita S, Shimada T, Yamai S, Ito T *et al.* Pulsed-field gel electrophoresis-based molecular comparison of *Vibrio cholerae* O1 isolates from domestic and imported cases of cholera in Japan. *J Clin Microbiol* 2000;38:424-6.
2. Cameron DN, Khambaty FM, Wachsmuth IK, Tauxe RV, Barrett TJ. Molecular characterization of *Vibrio cholerae* O1 strains by pulsed-field gel electrophoresis. *J Clin Microbiol* 1994;32:1685-90.
3. J, Nair GB, Kam KM, et al. Development and validation of a PulseNet standardized pulsed-field gel electrophoresis protocol for subtyping of *Vibrio cholerae.* Foodborne Pathog Dis. 2006;3:51–8. DOI: 10.1089/fpd.2006.3.51.
4. Mahalingam, S., Y. M. Cheong, S. Kan, R. M. Yassin, J. Vadivelu, and T. Pang. 1994. Molecular epidemiologic analysis of *Vibrio cholerae* O1 isolates by pulsed-field gel electrophoresis. J. Clin. Microbiol. **32:**2975–2979.
5. Nguyen BM, Lee JH, Cuong NT, Choi SY, Hien NT, Anh DD, et al. Cholera outbreaks caused by an altered *Vibrio cholerae* O1 El Tor biotype strain producing classical cholera toxin B in Vietnam in 2007 to 2008. J Clin Microbiol. 2009;47:1568–71. DOI: 10.1128/JCM.02040-08.
6. PulseNet PFGE Manual. Section 5a. August 2009. Rapid standardized laboratory protocol for molecular subtyping of Vibrio cholerae by pulsed-field gel electrophoresis (PFGE).
7. Safa A, Bhuiyan NA, Alam M, Sack DA, Nair GB. Genomic relatedness of the new Matlab variants of *Vibrio cholerae* O1 to the classical and El Tor biotypes as determined by pulsed-field gel electrophoresis. *J Clin Microbiol* 2005;43:1401-4.
8. Swaminathan, B., and G. M. Matar. 1993. Molecular typing methods, p. 26-50. In D. H. Persing, T. F. Smith, F. C. Tenover, and T. J. White (ed.), Diagnostic molecular microbiology: principles and applications. American Society for Microbiology, Washington, D.C.
9. Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH *et al.* Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol* 1995;33:2233-9.

3.14 Acknowledgements

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 4

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

4.2 Objectives:

a. 1st year:

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

b. 2nd year

- To determine the transmission route of *H.capsulatum* in Vietna and develop a collaborative network system for epidemiological study of histoplasmosis in Southeast Asia

c. 3rd year

- To announce the disease to clinical laboratory in the hospitals and Ministry of Health and the other public health offices in Vietnam.

4.3 Name of Researchers

National Institute of Hygiene and Epidemiology, Hanoi, Vienam (NIHE)

a. Thi Thu Ha HOANG MD., PhD – Bacteriology Department
b. Thanh Hai PHAM MSc
c. Thuy Tram NGUYEN MSc

National Institute of Infectious Diseases, Japan (NIID)

a. Hideaki OHNO MD., PhD – Department of Chemothorapy and Mycoses
b. Koichi TANABE PhD,
c. Takashi UMEYAMA PhD,
d. Satoshi YAMAGOE PhD,
e. Yoshitsugu MIYAZAKI MD., PhD,
f. Haruo WATANABE MD., PhD
g. BachMai hospital, Hanoi, Vietnam
h. Van Tien NGUYEN MD PhD – Infectious Diseases Department
i. Quang Tuan NGUYEN MD., PhD – Infectious Diseases Department

4.4 Affiliation

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

4.5 Sub-project title: Development of rapid diagnostic technique for detection of Histoplasmosis in Vietnam.

4.6 Summary

Histoplamosis is a common infection endemic in many regions of America, India, Africa and Asia, with sporadic cases also occurring throughout the world. The disease is caused by infection with *Histoplasma capsulatum (H.capsulatum)*, which is a dimorphic soil-borne fungus. The most common clinical presentation is asymptomatic. However, the symptoms of acute or epidemic histoplasmosis are high fever, cough, and asthenia. Histoplasmmosis also presents as an opportunistic infection, including AIDS. Histoplasmosis can be detected by culture, histopathology, serology and molecular methods. However, diagnosis depends on a high index of suspicion, knowledge of the clinical and epidemiologic features of the infection, and a thorough understanding of the uses and limitations of fungal cultural and serologic methods. A nested PCR has been developed for detection of histoplasmosis that amplifies a specific part of *H.capsulatum* M antigen gene. The PCR can be a useful additional tool for the diagnosis of Histoplamosis in Vietnam.

4.7 Purposes

Histoplasmosis is a fungal disease caused by infection with *Histoplasma capsulatum (H.capsulatum)*. Histoplasmosis, which can be acquired from soil contaminated with bird or bat droppings. Human infection occurs when airborne spores of *H.capsulatum* are inhaled. The most common clinical presentation is asymptomatic. The symptoms of acute or epidemic histoplasmosis are high fever, cough,

and asthenia. Histoplasmmosis also presents as an opportunistic infection in individuals with serious in underlying disease, including AIDS.

Histoplasmosis can be detected by culture, histopathology, serology and molecular methods. Although excellent laboratory methods are available, in many cases diagnosis is missed or delayed because histoplasmosis is not considered.

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 this area, however, the endemicity of the disease is not well studied, at present. In addition, the standard diagnostic tests for histoplasmosis are frequently not readily available, 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 or bird droppings. Furthermore, clinicians/researchers must be familiar with the uses and limitations of the current diagnostic tests available for fungal diseases.

The objective of the study is to apply a *histoplasma* specific PCR and serological diagnostic test in order to help rapid diagnsosis of this infectious disease and to improve laboratory capacity on diagnose of histoplasmosis by performing different methods in Vietnam.

4.8 Methods

4.8.1 Sample collection

During 6 months (from November, 2011 to March 2012), the study was carried out at Infectious Department, Bach Mai hospital, NIHE, some caves (Yen Bai province) and public areas in the North of Vietnam such as Dien Bien, Thanh Hoa, Hanoi. The clinical samples include bronchial washings, blood from suspected patients have been collected at Bach Mai hospital. On the other hand, the environmental samples: soil contaminated with bat, bird or chicken droppings inside the caves and public areas have also been collected by NIHE researchers. All samples were transported in a safety/cool box to laboratory and stored at -30^0C until analyzed.

Information on disease situation, history of disease, anti-fungi drug used, place to collect environmental samples has been filled in a set of questionnaire by the doctor/researcher with the accepted paper from patients and household member.

4.8.2 Microbiological methods

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

In briefly, a suspected/infected sample was cultured on surface of BHI with 1% glucose and/or blood agar 5% sheep blood (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 kept in room temperature/one month for further analysis. Preparation of DNA from clinical and environmental samples have been followed an 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. An in-house protocol for extraction of DNA

Serum 100 ul + lysis buffer 100 ul

↓

Proteinase K (finalconc. 60 ug/ml)

↓

55°C, 60 min

↓

95 °C, 10 min

↓

Cool down

↓

Add wastase (β-glucanase)

↓

37 °C, 60 min

↓

12,000xg, 10min

↓

Supernatant 10 ul for PCR

The template DNA was tested 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 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 95^{0}C for 5min; 40 x (94^{0}C for 1min followed by 60^{0}C for 1min and 72^{0}C for 1min); 1 x 72^{0}C for 5 min; cool to 4^{0}C. 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.

Blood sample was aslo be performed by serological test following the manufacture's instruction of Histoplasma DxSelect™ 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 a spectrophotomeric reading of optical density (OD). ELISA results are compared with reference cut-off OD readings by an ELISA reading machine.

4.9 Results

During the study time, 149 serum samples and bronchial washings have been collected from unknown fever patients in Bach Mai hospital. Among them 15 serum samples are patient with HIV positive. At the same time, 66 soil samples from the caves in YenBai province and public areas in the north of VietNam have been collected.

Five bronchial washing and serum samples were cultured on Brain Heart Infusion (BHI) with 1% glucose and BHI containing 5% horse blood and incubated at $37^0C/30^0C$ for at least one month. The culture results are still negative, at present.

Serum samples from 144 suspected patients were tested for antibody reactivity by an ELISA. The ELISA positive obtained in 26 serum samples and three of them are patients with HIV positive (see detail in figure.1).

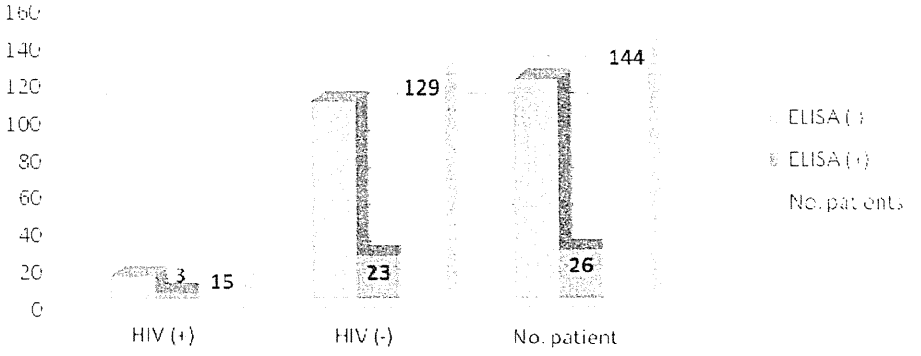
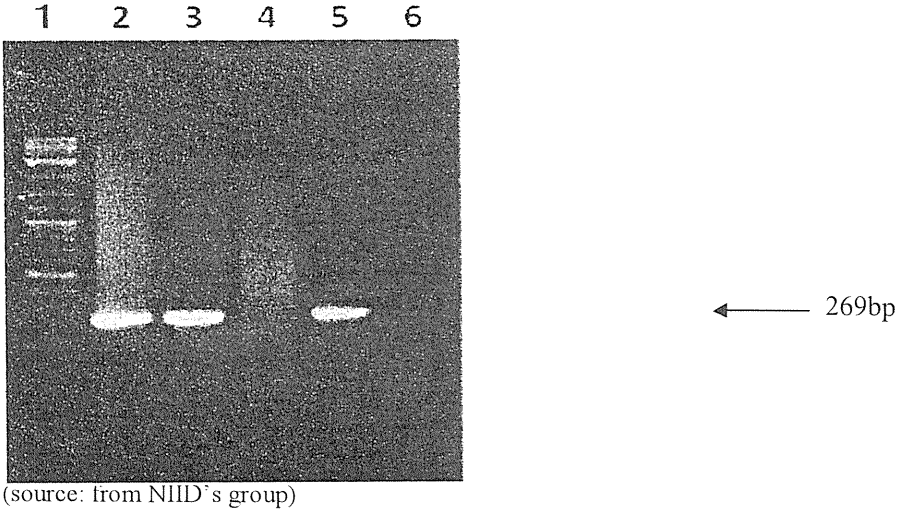


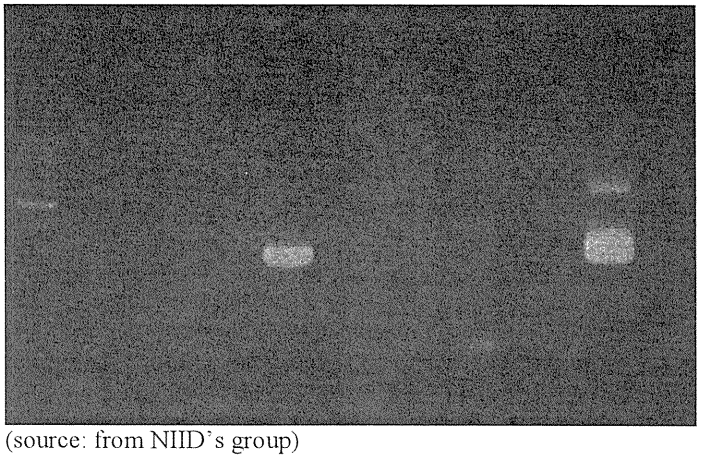
Figure 1. ELISA results from serum samples with Histoplasma DxSelect™ kit

In comparing with a *H.capulatum* specific PCR, the results from both clinical and environmental samples were negative.



(source: from NIID's group)

Figure 2. Nested PCR with a mixture of Msp1F/Msp2R; Msp2F/Msp3R primers from clinical samples
Lane 1: 100 bp molecular size marker (Invitrogen)
Lane 2-3: clinical samples
Lane 4: serum samples (healthy person)
Lane 5: positive control
Lane 12: negative control



(source: from NIID's group)

Figure 3. Nested PCR with a mixture of Msp1F/Msp2R; Msp2F/Msp3R primers from soil samples
Lane 1: 100bp molecular size marker (Invitrogen); Lane 10: Negative control
Lane 2-9: soil samples Lane 11: Positive control

4.10 Discussions

The diagnosis of histoplasmosis is based on the results of careful clinical evaluation and associated laboratory tests. The fungus can be cultured from different sources such as blood, respiratory secretions, bronchial washings. Isolation of *H. capsulatum* from clinical samples provides a definitive diagnosis, but this fungus frequently fails to grow under artificial culture conditions. In addition, the incubated fungus time usually takes between 2 and 6 weeks, introducing undesirable delay in diagnosis and therapy. In this period, we have cultured five clinical samples, but the results are still in awaiting time. Moreover, the cultured plates are showing with many common bacteria grown. Thus, we recommended the BHI culture medium should be added antibiotics for culturing the clinical samples. That is an evidence for showing that culture method is time-consuming and costly and is not useful for treatment purpose. In the literature, cultures are negative in most mild form of histoplasmosis and the *Histoplasma* cultured positives are around 10%. In addition, there is no published data on isolation of *H.capsulatum* from environmental samples *e.g.* soil samples. Although, isolation of organisms provides the strongest evidence but is not consider as a standard method for infection with *H.capsulatum.*

In such cases, serologic tests are indicated for detection of antibodies against M antigen. These tests are faster than culture, but they have 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 serological method is available for the screening and evaluation of the prevalence of histoplamosis, only. Our result showed, 18% of unknown fever patients were positive with ELISA is high to compare with other Asia countries such as Japan. However, the number of HIV positives in total serum samples is only 10%. Thus, we need to collect more clinical samples, especially in opportunistic patients, including AIDS. It could be given a higher result than 18%.

Several reports suggested that PCRs might improve the accuracy of identification of *H. capsulatum* in clinical samples such as tissues, body fluids. Due to, the recovery of *H. capsulatum* from soil coupled with the finding of well-documented cases of histoplasmosis, improved tests for the detection of *histoplasma* in environmental sites are also needed. In this study, we developed a nested PCR with the specific primers for detection of *H.capsulatum* in both clinical and environmental samples. The design of the primers have been published but modified by NIID researchers for increasing the sensitivity and specificity of PCR. Because of the study time is very limited we have been analyzed 20 clinical and environmental samples by nested PCR, only. All samples were negative by PCR but the positive and negative controls were presented clearly. The remains samples will be done in the next period. PCR, however, has been showing a rapid method for detection of *H.capsulatum* and performing well in

Vietnam. Therefore, the method could be useful for further studies in order to demonstrate the trend of disease.

In conclusion, comprehensive mycological, serological, molecular methods for detection of *H.capsulatum* from both clinical and soil samples are indicated in order to investigate the prevalence and incidence of histoplamosis and to map out the endemic areas of the disease in Asia.

4.11 Publications

None

4.12 Reference

1. Romano C, Castelli A, Laurini L, et al. Case report. Primary cutaneous histoplasmosis in an immunosuppressed patient. Mycoses 2000; 43: 151_154.
2. Wheat, L. J. (2001). Laboratory diagnosis of histoplasmosis: update 2000. Semin Respir Infect 16, 131–140.
3. Dano MVC, Hajjeh RA. The epidemiology of histoplasmosis: a review. Sem Resp Inf 2001; 16: 109_118.
4. Kauffman CA. Fungal infections in older adults. Clin Infect Dis 2001; 33: 550_555.
5. Wheat, L. J., Garringer, T., Brizendine, E.&Conolly, P. (2002). Diagnosis of histoplasmosis by antigen detection based upon experience at the histoplasmosis reference laboratory. Diagn Microbiol Infect Dis 43.
6. Calza L, Manfredi R, Donzelli C, et al. Disseminated histoplasmosis with atypical cutaneous lesions in an Italian HIV-infected patient: another autochtonous case. HIV Med 2003; 4: 145_148.
7. Wheat, L. J. & Kauffman, C. A. (2003). Histoplasmosis. Infect Dis Clin North Am 17, 1–19.
8. Kauffman CA. Histoplasmosis: a clinical and laboratory update. Clin Microbiol Rev 2007; 20: 115_132. 29–37.
9. Murata, Y., Sano, A., Ueda, Y., Inomata, T., Takayama, A., Poonwan, N., Nanthawan, M., Mikami, Y., Miyaji, M., Nishimura, K. and Kamei, K. 2007. Molecular epidemiology of canine histoplasmosis in Japan. *Med. Mycol.* **45**: 233–247. 18.
10. Ryosuke KOBAYASHI, Fumihiko TANAKA, Atsusi ASAI, Yumiko KAGAWA, Teruo IKEDA and Kinji SHIROTA. First Case Report of Histoplasmosis in a Cat in Japan. *J. Vet. Med. Sci.* 71(12): 1669–1672, 2009.

4.13 Acknowledgements

"This work was supported by a grant-in-aid of Ministry of Health, Labor and Welfare, the Government of Japan (H23-Shinkou- shitei-020)"

We would like to thank to Dr. Nguyen Kieu Anh's group in Virology Department for helping us to collect soil samples from caves in YenBai province. Sincerely, thank to Department of International Cooperation, NIHE for administration support.

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 Objectives:

a. ***1st year*** : To perform a differential diagnosis tools for detection of *Leptospira* from samples such as the microscopic agglutination test (MAT; gold standard method), recombinant protein (LigA)-based IgM ELISA, a whole cell-based IgM ELISA, and molecular methods (e.g. PCR, loop-mediated isothermal amplification – LAMP) in Vietnam

b. ***2nd year:*** To determine the transmission of disease between human, animals/environment (contaminated with animal urine) by those tests.

c. ***3rd year:*** To investigate the prevalence of leptospirosis among patients with fever of unknown origin in northern of Vietnam

5.3 Name of Researchers

National Institute of Hygiene and Epidemiology, Hanoi, Vietnam (NIHE)

a. Dr. Thi Thu Ha HOANG – Bacteriology Department
b. Thanh Hai PHAM, MSc
c. Thuy Tram NGUYEN, MSc

National Institute of Infectious Diseases, Tokyo, Japan (NIID)

a. Dr. Nobuo KOIZUMI - Department of Bacteriology
b. Dr. Makoto OHNISHI

Bach Mai hospital, Hanoi

a. Dr. Thanh Thuy PHAM – Infectious Diseases Department

Preventive Medicine Center, ThanhHoa, Vietnam

a. Dr. Dinh Ngu HA

5.4 Affiliation

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

5.5 Sub-project title: Establishment of laboratory diagnosis for Leptospirosis in Vietnam.

5.6 Summary

Leptospirosis is a transmissible disease of animals and humans caused by infection with any of the pathogenic members of the genus *leptospira.* Laboratory diagnosis of leptospirosis are designed to detect anti-leptospiral antibodies and to detect leptospiral antigens, or *leptospiral* DNA in animal and human. 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 leptospirosis in the country is lacking except Mekong Delta region. To describe an overall infection of *leptospira* in Vietnam, we have developed a new serological diagnostic method by using a recombinant protein – based IgM to compare with a whole cell-based IgM for detection of *leptospiral* antibodies. In addition, a *flaB*-nested PCR using a mixture specific primers in the 16S region of ribosomal RNA have performed for detection of *leptospiral* DNA from blood and urine samples. To be adapted for a new mordern technique, we aslo applied the loop-mediated isothermal amplification (LAMP) method for the detection of *leptospira* DNA directly from urine samples. These methods are simple and helpful for diagnosis and surveilance of leptospirosis in Vietnam. However, the sensitivity and specificity of those tests need to be evaluated by comparing with culture and microscopic agglutination test (MAT) in the next period of the project.

5.7 Purposes

Leptospirosis, a zoonosis with preference for warmer climates, caused by infection by pathogenic Leptospiral species. Transmission usually results from direct or indirect exposure to the urine and other body fluids of infected animals. Indirect exposure occurs via contact with contaminated water and soil.

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 the microscopic agglutination test facilities. In addition, molecular methods are aslo useful for identification of bacteria from resevoir animal as well as establish the transmission of disease disease between human, animal and environment.

Since the 1930s Vietnam is known to be endemic, with the first case of Leptospirosis in North Vietnam was in 1937. Hight rate of seropositivity against leptospira in Vietnamese general population has been observed in previously, ranging from 10%-30% (Van etal., 1998). However, the study did not concern about the risk factors as well as doing a resevoir animal survey. There has been, up to now, a lack of study on prevalance of Leptospirosis in Vietnam, especially in Thanh Hoa province.

Thanh Hoa province is a large area, with warm climate, high rainfall, ample sunlight, which are favorable for developing agriculture, rearing forestry and fishery. That is an optimal conditions for transmission of leptospirosis to human. In addition, one of the serovars included in the major pathogenic species has been isolated in this area (unpublished data). Howver, no more information is provided on laboratory diagnosis tools for detection of *leptospira* in both provincial and institutional level.

In order to improve laboratory capacity for detection of leptospira, in the present study, we develop a new ELISA by using recombinant protein –based IgM to compare with a whole cell-based IgM, and a molecular methods for detection of *leptospira* DNA directly from clinical samples for early diagnosis.

5.8 Methods.

5.8.1 Study design

This study was conducted in Bach Mai hospital, ThanhHoa province and NIHE in the five months period (from November 2011 to March 2012) The hospitalized patients with fever of unknown origin at Bach Mai, Hanoi have been selected by the doctors in the hospital. Blood samples were collected from a patient with acute febrile illness, with headache, myalgia, and prostration associated with any of the following: Conjunctival suffusion/haemorrhage; meningial irritation; anuria/Oliguria/ haematuria/ proteinuria; hemorrhage – intestinal bleeding, lung bleeding or purpuric rash; cardiac arrhythmias/ failure; jaundice.

Urine samples from animals such as pigs and cattle were collected in Yen Dinh and Nhu Thanh sub-urban, ThanhHoa. A kidney tissue sample from animal should be collected (if possible).
All samples were transported in a safety/cool box to laboratory and stored at -30^{0}C until analyzed.
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 collected by the doctors/researchers with the accepted paper form patients/participants.

5.8.2 Microbiological methods

The serological methods employed in this study are the microscopic agglutination test (MAT; gold standard method), a whole cell-based IgM ELISA. In addition to these established method, we employ a recombinant protein (LigA)-based IgM ELISA, which we have developed recently, and evaluate the usefulness of the IgM ELISA. We also detect *leptospira* DNA from the same serum samples 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 5 mins in order to remove blood cells or, cell debris and protein precipitates. The supernatant was transferred to a new tube and centrifuged with 13,000×*g* at 4^{0}C for 20 mins. DNA was extracted from the pellet by using DNeasy Tissue Kit (Qiagen) according to manufacture's protocol.

In order to amplify DNA in blood and urine samples, L-*flaB*1/M-L-*flaB*2 primers were employed as described by NIID's group. The reaction with L-*flB* F1 5'-TGT GCA CAA GAC GAT GAA AGC -3' (23 Nu) and L-*flB* R1 5'- AAC ATT GCC GTA CCA CTC TG -3' (22 Nu) primers consisted of 94^{0}C for 1 min, 25 cycles at 94^{0}C for 10s, 50^{0}C for 30s, 72^{0}C for 1 min, and 72^{0}C for five additional minutes.

The reaction with M-L-*flaB* F2 5'-TGT GCA CAA GAC GAT GAA AGC -3' (21 Nu) and M-L-*flaB* R2 5'- AAC ATT GCC GTA CCA CTC TG -3' (20 Nu) primers consisted of 94^0C for 1 min, 30 cycles at 94^0C for 10s, 50^0C for 30s, 72^0C for 50s, and 72^0C for seven additional minutes. These primers are corresponding to a conserved sequence in the 16S region of 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 20 mins before staining with SYBR safe DNA stain.

A loop-mediated isothermal amplification (LAMP) method for the detection of *Leptospira* DNA directly from urine samples is also performed. A urine sample is subjected to centrifugation (100×*g*, 5 min) if some precipitates are seen in it. The supernatant after centrifugation is boiled for 10 min at 100^0C. The boiled sample is subjected to centrifugation (13,000×*g*, 1 min) and the resulting supernatant is used as a template for *Leptospira rrs* LAMP with the condition of 65^0C/60 mins and 95^0C/5 mins.

5.9 Results

We have got 180 serum samples from unknown fever patients in BachMai hospital. At the same time, 30 urine samples and kidney tissues have been collected from animals in Thanh Hoa province.

All blood samples were tested by ELISA with whole-cell based IgM and a recombinant protein (LigA)-based IgM. 13 serum samples were positive by both ELISAs, and 5 samples were positive by E LISA with whole-cell based IgM, only (see detail in Figure 1).

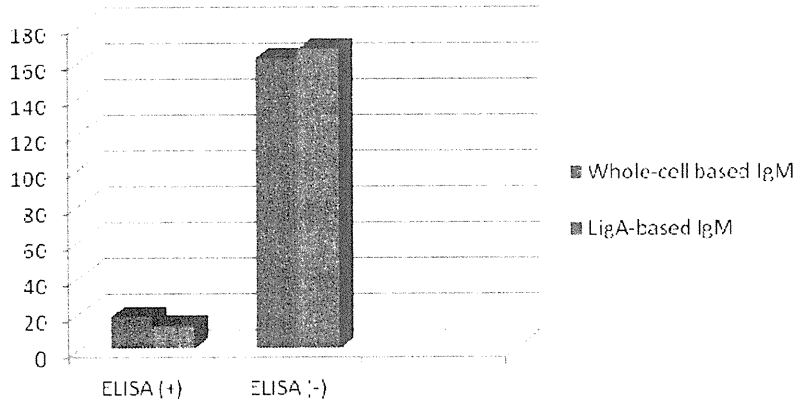


Figure 1. ELISA results from serum samples with Histoplasma DxSelect™ kit

During the study period, we haven't got any suspected cases in animal. The culture method was applied by using the tissues kidney samples from health dogs. Due to the MAT needs to use *leptospira* cultures (1-2 x 10^8 cell/ml), the MAT has not performed in our laboratory, yet.

The urine samples were extracted DNA and analyzed by nested PCR. We used a recombinant positive control with 400bp that provided by NIID's researcher group. However, PCR results from both blood and urine samples were negative (showed in Fig. 2).

25 urine samples from animals were also negative by LAMP method.

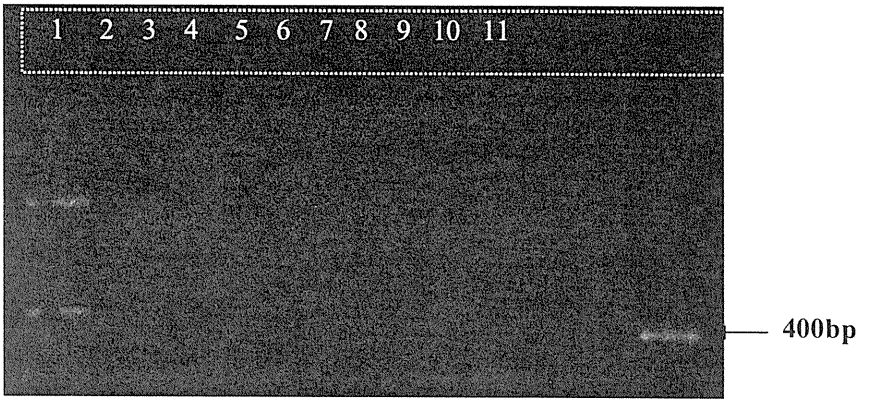


Figure 2. Nested PCR with a mixture of L-*flB*F1/L-*flB* R1; M-L-*flB*F2/M-L-*flB* R2 primers from clinical samples

Lane 1: 100 bp molecular size marker (Invitrogen)
Lane 2 – 9: clinical samples
Lane 10: Negative control
Lane 11: Positive control

5.10 Discussion

The use, interpretation, and value of laboratory diagnostic for leptospirosis vary with clinical history of the animal/human, the duration of infection, and the infecting dose/serovar. The MAT using live antigen is most widely used serological test. The sensitive of the test can be improved by using local isolates rather than reference strains, but the reference strains assist in the interpretation of results between laboratories. The MAT is very useful in diagnosing acute infection (by demonstration of a four-fold change in antibody titres in paired acute). However, MAT has limitations in diagnosis of chronic infection because of the titres are often very low. In addition, MAT using live antigen that means the laboratory has to re-culture strains frequently. It depends on the laboratory capacity because the isolation of leptospires is time-consuming and is a task for specialized reference laboratories, only. Therefore, it should be performed in NIHE laboratory in the next period.

Another serological test, ELISA can also be useful for detection of antibodies against leptospries In general, ELISAs are quite sensitive, but lack the serovar specific of the MAT. Many ELISAs have been developed using a number of different antigen preparations. In this study, we used a recombinant protein-based to compare with a whole cell-based for an ELISA to identify IgM level in the blood samples. Totally, we got 10% of ELISA positive from suspected patients in BachMai hospital. The positive results of ELISA using recombinant protein-based and whole cell-based are 13/180 and 18/180, respectively. The difference result between two kinds of antigens was 5 positive blood samples, only. Therefore, we need to compare with other tests or tested with a different number of samples.

Leptospiral DNA can be demonstrated in tissues, body fluid 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 PCR result, showed very clear band of the controls and all samples were negative. It means that, we have succeeded to perform the assay in the laboratory. However, we need to mention in reducing amount of DNA template for PCR assay.

In this time, we have applied LAMP as a rapid method for detection of *leptospira* infection. However, the method should be evaluated in the further studies.

In conclusion, the laboratory diagnosis for detection of leptospirosis has been established in Vietnam. However, selecting an available diagnostic test is depends on the purpose of testing and on the tests or expertise available in the area.

5.11 Publications

None

5.12 Reference

1. Van CT, Thuy NT, San NH, Hien TT, Baranton G, Perolat P. Human leptospirosis in the Mekong delta, Vietnam. Trans R Soc Trop Med Hyg. 1998 Nov-Dec;92(6):625-8.
2. Rebeca Plank, Deborak Dean. Overview of the epidemiology, microbiology, and pathogenesis of *Leptospira* spp. in humans. Microbes and Infection, 2000. 1265 – 1276.
3. Revital Kariv, Robert Klempfner, Ada barnea and et al. The changing Epidemiology of Leptospirosis in Israel. Emerging Infectious Diseases, Vol 7, No 6, November-December 2001.
4. Boqvist S, Chau BL, Gunnarsson A, Olsson Engvall E, Vågsholm I, Magnusson U. Animal-and herd-level risk factors for leptospiral seropositivity among sows in the Mekong delta, Vietnam. Prev. Vet. Med. 2002 Mar 14;53(3):233-45.
5. Boqvist S, Thu HT, Vågsholm I, Magnusson U. The imfact of Leptospira seropositivity on reproductive performance in sows in Southern Vietnam. Theriogenology. 2002 Oct 15;58(7):1327-35.
6. Phran Phraisuwan, Ellen A.Spotts Whitney, Piyanit Tharmaphornpilas, et al. Leptospirosis: Skin Wounds and control Strategies, Thailand, 1999. Emerging Infectious Diseases, Vol.8, No 12, December 2002.
7. Kanti laras, Van CB, Khanthong Bounlu and el al. The importance of Leptospirosis in southeast asia. Am J.Trop.Med.Hyg., 67(3), 2002,pp.278-286.
8. Kanti Laras, Cao Bao Van, Khanthong Boulu, Nguyen Thi Kim Tien and et al. The importance of Leptospirosis in Southeast Asia. Am.J. Trop.Med.Hyg., 67 (3), 2002, pp. 278-286.
9. Prevalence of and risk factors for serum antibodies against Leptospira serovars in US veterinarians. J Am Vet Med Assoc. 2009 Apr 1;234(7):938-44.
10. de Abreu Fonseca C, Teixeira de Freitas VL, Caló Romero E, Spinosa C, Arroyo Sanches MC, da Silva MV, Shikanai-Yasuda MA. Polymerase chain reaction in comparison with serological tests for early diagnosis of human leptospirosis. Trop Med Int Health. 2006 Nov;11(11):1699-707.

5.13 Acknowledgements

"This work was supported by a grant-in-aid of Ministry of Health, Labor and Welfare, the Government of Japan (H23-Shinkou- shitei-020)"

We would like to thank to the Department of International Cooperation, NIHE for their administrative support.

Research 6

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

6.2 Objectives: To improve laboratory capacity of NIHE researcher and also established a strong net work for Anthrax disease in the north of Vietnam.

6.3 Name of Researchers

National Institute of Hygiene and Epidemiology, Hanoi, Vienam (NIHE)
a. Thi Thu Ha HOANG MD., PhD – Bacteriology Department
b. Thanh Hai PHAM MSc
c. Thuy Tram NGUYEN MSc
National Institute of Infectious Diseases, Japan (NIID)
a. Akiko OKUTANI MD., PhD – Department of Veterinary Science
b. Satoshi INOUE DVM., PhD
DienBien Preventive Medicine Center
a. Ngoc Hung DOAN MD., Director
b. Trong Canh LE, MD., Deputy Director , Epidemiology Department

6.4 Affiliation

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

6.5 Sub-project title: Detection of Anthrax by applying differential diagnostic tools in Vietnam

6.6 Summary

Anthrax is an acute bacterial zoonoses caused by a Gram-positive bacterium, *Bacillus anthracis*. Human beings almost invariably contract anthrax from animals. The infection is clearer by showing the evidence of the transmission sources. Anthrax incidence in humans is frequently occurred in Vietnam, recent years. However, the laboratory diagnostic is still lacking. In addition, anthrax network for reporting and collecting samples is not in good connection. Therefore, we applied differential diagnosis tools for detection of *B.anthracis* from clinical and environmental samples. A basic method such as culture, microscopic, biochemical tests have performed in the same time with other molecular methods such as PCR assays. The PCRs are rapid and sensitive methods for detection of *B.anthracis* in both clinical and environmental samples. In addition, a multiple locus variable tandem repeat can be applied for a further molecular epidemiology studies in Vietnam.

6.7 Purposes

Bacillus anthracis (*B.anthracis*) 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. The soil-forming condition of *B.anthracis* is the most suitable for producing inhalation airborne anthrax, meaning that *B.anthracis* needs to be subject to continuous epidemiological surveys of the environment because it can survive in the soil for a long period of time in its spore form.

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. Most of infected cases have been identified by clinical symptoms only and related to eat infected animals. Otherwise, in these areas, sporadic coetaneous cases haven't contacted 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 can be affect to community and environment.

There are a range of laboratory techniques available for the diagnosis of anthrax in human and herbivores, including microscopy, culture and PCR. However, microscopy and culture may become false-negative results if the smear is prepared from animals that have been dead more than about 6h, and

contamination cultures when samples are collected from long-dead case or environment. A number of PCP assays have been described for the identification of *B.anthracis* from different kind of samples. However, PCRs detection of *B.anthracis* in infected animal or patient specimens are easy but it is difficult in environmental resource e.g. soil and have not use as diagnostic tools in laboratory in Vietnam, yet.

In this study, we used different methods for detection of *B.anthracis* in order to improve laboratory capacity of NIHE researcher and also established a strong net work for Anthrax disease in the north of Vietnam.

6.8 Methods.

6.8.1 Sample collection

A total 10 eschar and vesicular fluid samples were collected from suspected patients. In the same time, 70 soil samples have aslo collected around the cage of the cow/goat and suspected patient houses in Dien Bien.

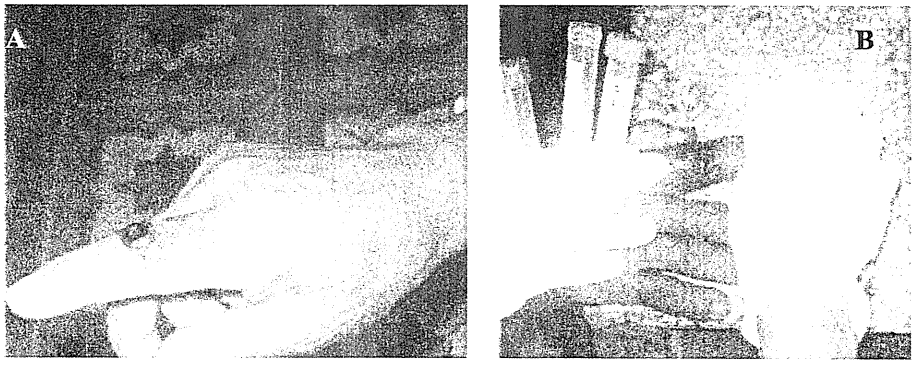


Fig. 1 (A&B). Collection of sample from Anthax suspected case in Dien Bien Feb. 2012

Samples were transported to local laboratories and NIHE following the Anthrax national standard operating procedure.

Information on the history of exposure to infected animals/environment contaminated with animal discharge, age, gender, social – economic, education has been asked by the medical staff with the accepted paper from patients.

6.8.2 Sample preparation

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

6.8.3 Bacterial strains:

Two strains of *B.athracis* 17JB, 34F2 DK (pXO1 -/pXO2+) were used (kindly provided by National Institute of Hygiene and Epidemiology frozen strains Bank).

6.8.4 Culture and DNA extraction:

B.anthracis 17JB and 34F2 DK were cultured on 5% sheep blood agar and (BA) and nutrient agar (NA) for re-selected of *B.anthracis* isolate strains following overnight incubation at 37^0C. The suspected colonies will be identified by microscopic and biochemical tests. Then, one colony of *B.anthracis* was cultured in 2ml of trypticase soy broth at 37^0C/overnight for DNA extracting purpose.

DNA was extracted from 500µl of the overnight cultured *B.anthracis* using QIAamp® DNA mini kit. The DNA was used as positive controls.

Ten fresh clinical samples were processed and extracted by using QIAamp® DNA mini kit.

70 soil samples were extracted by PowerSoil™ DNA Isolation kit (MOBIO) (according to manufacturer's instruction). DNA extracts were stored at minus 20^{0}C until running PCR.

6.8.5 Spore preparation

Spores were prepared from *B.anthracis* 34F2 DK by method of Fasanella et al (2003). Spores were inactivated by incubation with asuspension of 50% methanol and 50% saline at 37^{0}C for 1 h . Then, spores were washed three time with saline and the pellet was suspended. Spore stock solutions were kept in the separate 5ml tube screw cap and putted in the stainless steel box. The stainless steel box were stored at minus 80^{0}C in BSL3.

For the PCR assay, 200 µl of spore suspension was heated to 120^{0}C/60mins then mixed with one soil samples which was collected in National Institute of Hygiene and Epidemiology (NIHE). The soil sample included spores was extracted DNA by using by PowerSoil™ DNA Isolation kit.

6.8.6 PCR

Detection of *B.anthracis* by multiplex 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)

Primers	Target gene	Sequence	Lengh of amplicon (bp)
PA5 PA8 (WHO)	*pag*	TCC TAA CAC TAACGA AGT GAA GGA CTG GTA GAA GGATAT ACG GT	603 bp
PA7 PA6 (NIID)	*pag*	CTACAGGGGATTTATCTATTCC ATTGTTACATGATTATCAGCGG	151 bp
CAP1234 CAP1301 (WHO)	*cag*	CTG AGC TAA TCG ATA TG TCC CAC TTA CGT AAT CTG AG	846 bp
MO11 MO12 (NIID)	*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^{0}C for 5min; 30 x (94^{0}C for 30s followed by 55^{0}C for 30 s and 72^{0}C for 55s); 1 x 72^{0}C for 5 min; cool to 4^{0}C. 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.

6.9 Results

The 17JB and 34F2DK *B.anthracis* strains were grown well on sheep blood agar. Six clinical samples were positive by culturing.