- Place slides in a covered, moist chamber, and incubate at 35^{0}C 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 vó 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)

- Thi Thu Ha HOANG MD PhD, ThanhHai PHAM MSc, Thuy Tram NGUYEN MSc – Bacteriology Department – NIHE
- Van Tien NGUYEN MD PhD – Infectious Diseases Department - BachMai hospital, Hanoi, Vietnam
- Ass.Prof. Thai Son NGUYEN MD, PhD – ClinicalMicrobiology Department - 103 Military Hospital, Hanoi, Vietnam
- Hideaki OHNO MD PhD – Department of Chemotherapy and Mycoses - National Institute of Infectious Diseases, Japan (NIID)
- Koichi TANABE PhD,
- Takashi UMEYAMA PhD,
- Satoshi YAMAGOE PhD,
- Yoshitsugu MIYAZAKI MD PhD,
- Haruo WATANABE MD PhD

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^{0}C 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.

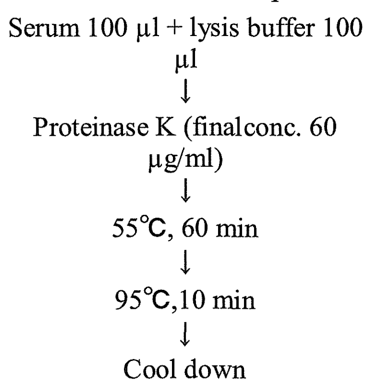
Microbiological methods

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



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.

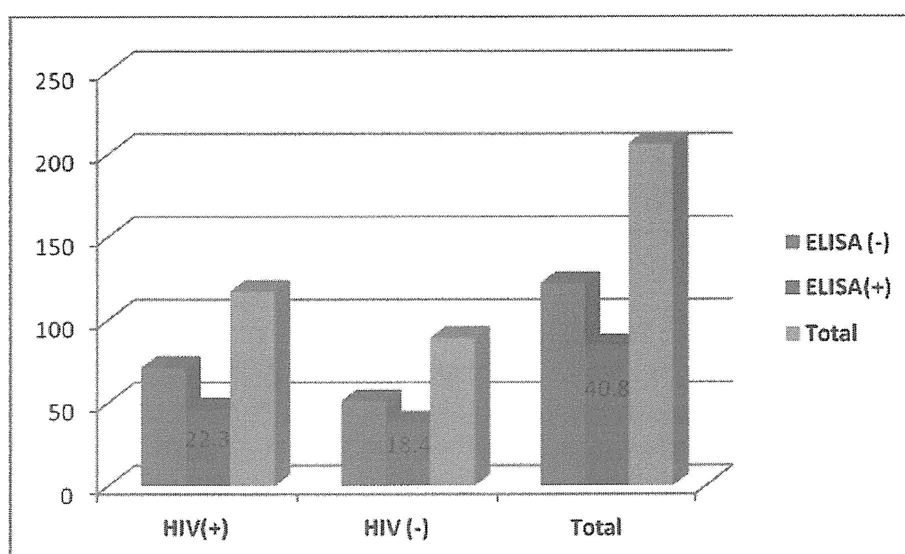
4.10 Results

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.

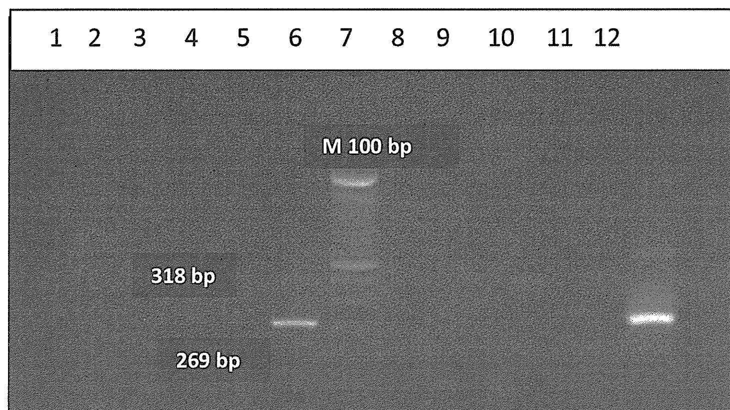
Figure 1. ELISA results of lung infected patients by HistoplasmaDxSelect™ kit



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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We would like to thank the International Cooperation Department, 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 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

- **Promotor:** Prof. Tran Hien NGUYEN - National Institute of Hygiene and Epidemiology, Hanoi, Vienam (NIHE)
- Thi Thu Ha HOANG MD PhD, ThanhHai PHAM MSc, Thuy Tram NGUYEN MSc – Bacteriology Department – NIHE
- Nhu Duong TRAN MD, PhD – Epidemiology Department – NIHE
- ThanhThuy PHAM MD PhD – InfectiousDiseasesDepartment - BachMai hospital, Hanoi, Vietnam
- Ass.Prof. Thai Son NGUYEN MD, PhD - ClinicalMicrobiologyDepartment- 103 MilitaryHospital, Hanoi, Vietnam
- DinhNgu HA MD – ThanhHoa Preventive Medicine Center
- 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).

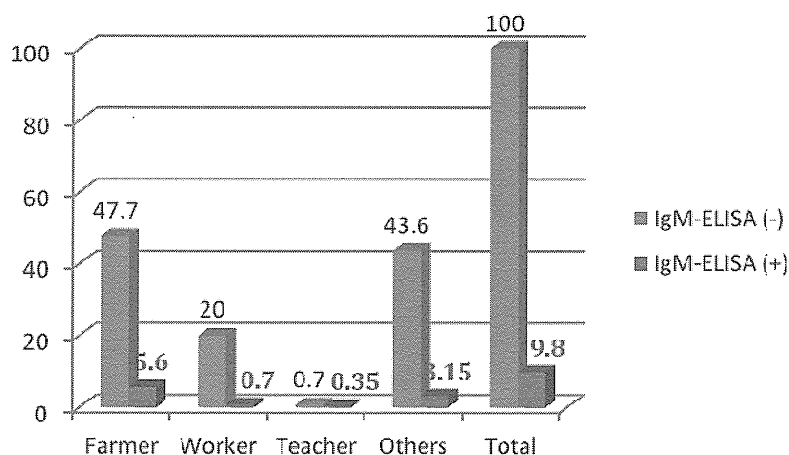

Fig.1. IgM-ELISA with recombinant antigen LigA results from serum samples of patients

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 laboratory testing.

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

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

Thi Thu Ha HOANG MD PhD, ThanhHai PHAM MSc, Thuy Tram NGUYEN MSc – Bacteriology Department – NIHE

Nhu Duong TRAN MD, PhD – Epidemiology Department –NIHE

Ngoc Hung DOAN MD, Trong Canh LE MD – Epidemiology Department – Dien Bien Preventive Medicine Center

Akiko OKUTANI DMV, PhD,

Satoshi INOUE DMV, PhD – Department of Veterinary Science - National Institute of Infectious Disease, Tokyo, Japan

6.4 Affiliation

National Institute of Hygiene and Epidemiology, Vietnam.

National Institute of Infectious Diseases, Japan.

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^{0}C 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^{0}C 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^{0}C. 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^{0}C 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^{0}C 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^{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.

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^{0}C for 2 min; 30 x (94^{0}C for 30s followed by 52^{0}C for 30 s and 68^{0}C for 1 min); 1 x 68^{0}C 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