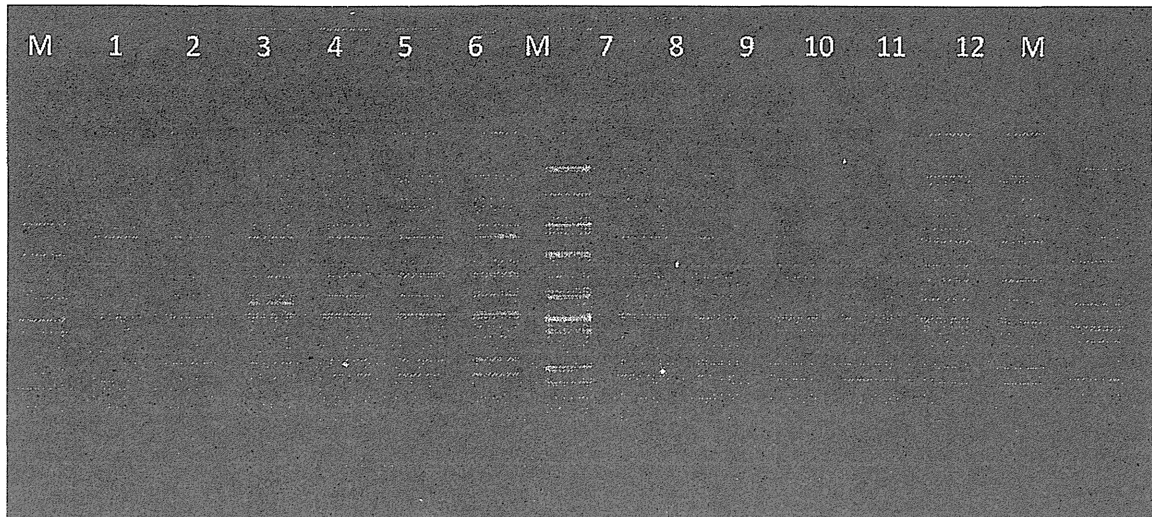
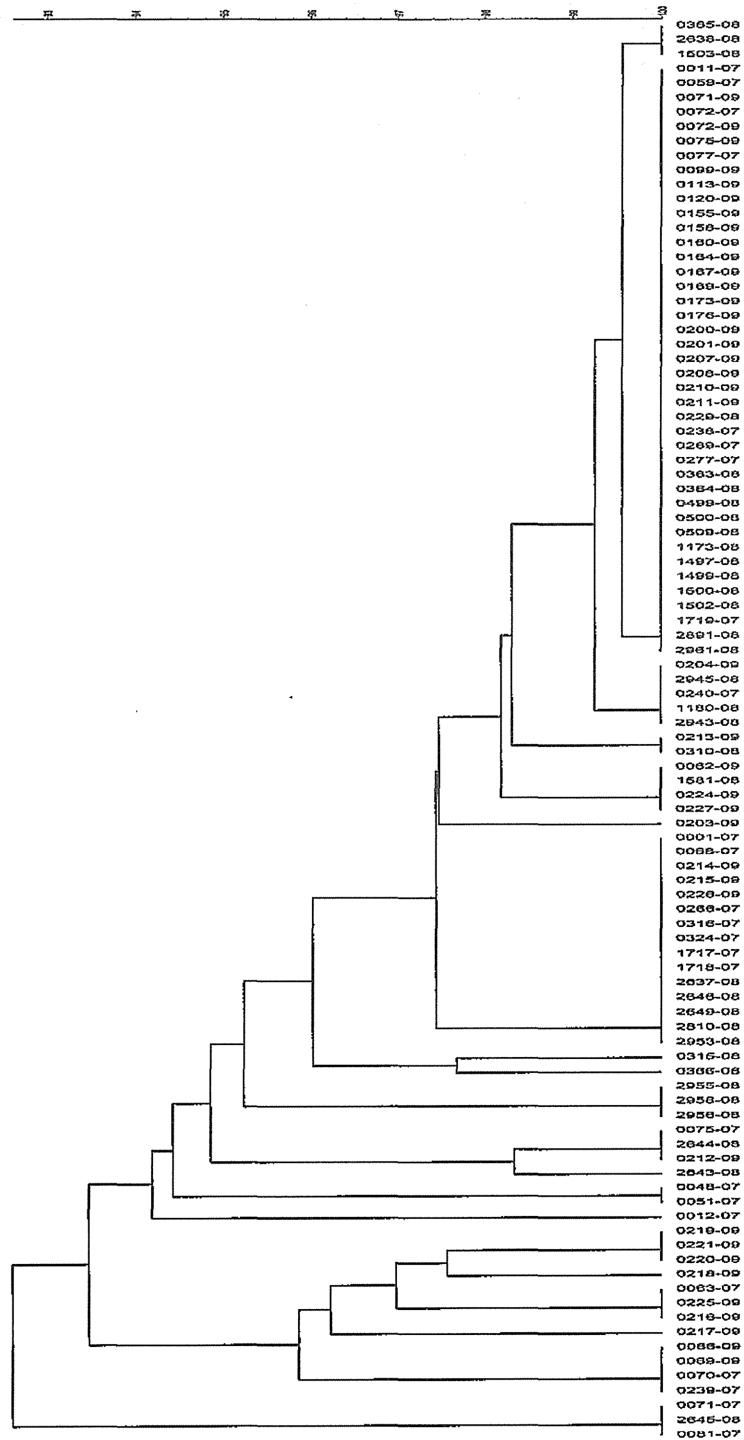


FIG.2. Comparison of Representatives PFGE patterns of *V. cholerae* O1 clinical strains and enviromental strains. Lanes 1-4: *V. cholerae* O1 enviromental strains (VCE11-07, 12-07, 81-07, 217-07). Lanes 5-12: *V. cholerae* O1 clinical strains (VC0225-09, 0227-09, 59-07, 269-07, 363-08, 2691-08, 169-09, 213-09) show representatives PFGE another patterns of type.

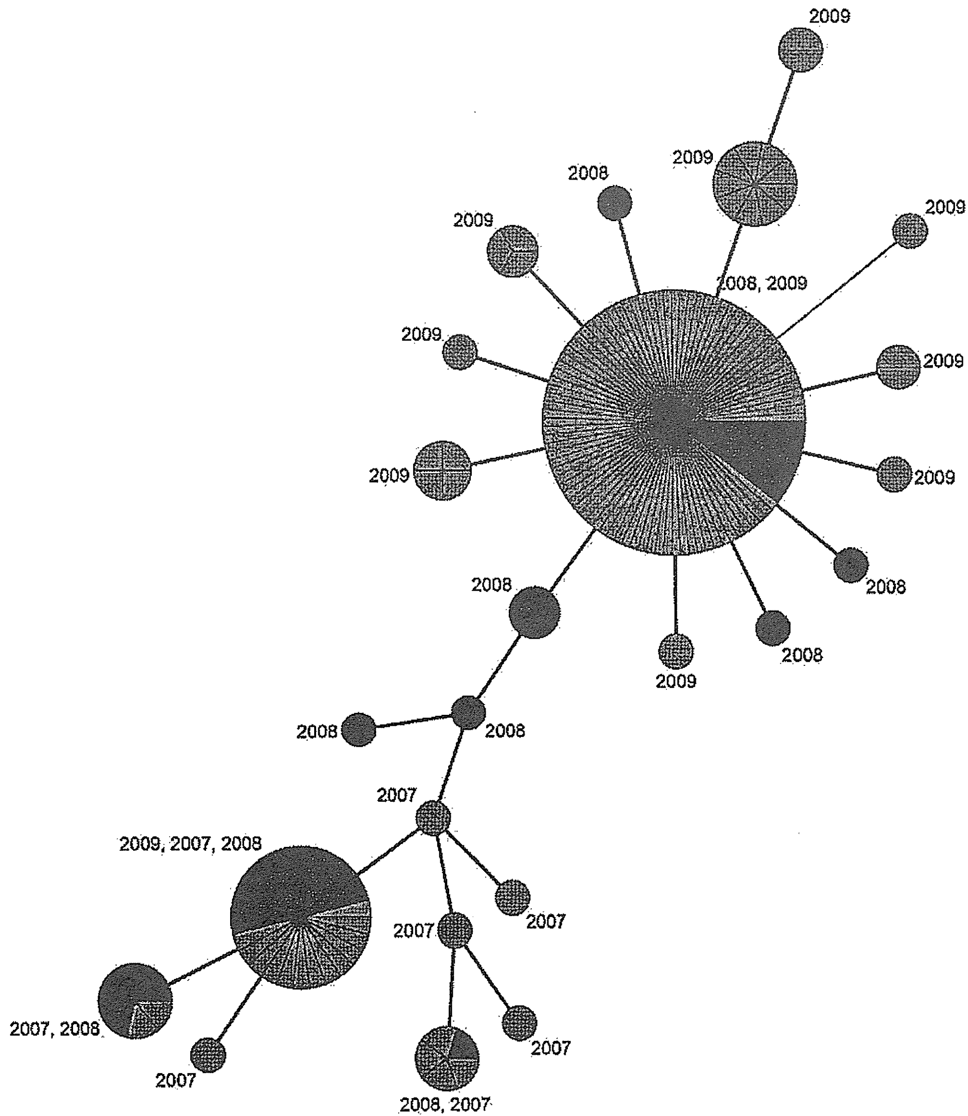
One hundred of *V. cholerae* O1 isolates collected from different provinces during various times (in period 2007 to 2009) exhibited very similar, although not entirely identical, PFGE banding patterns which suggest that they are clonal. The numerical similarities between isolates, as defined by Dice 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 were similar from those of the enviromental isolates.

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



3.3. Result of MLVA

MLVA revealed 25 subtypes of the isolates during the period of 2007-2009.
Cholera in 2007-2009 seems to be caused by a single clone with small variations of subtypes.



3.4. MPN-mPCR 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 44 of water samples of tested were all negative for V.O1,*ctx A* and 11 of them positive for *ToxA* 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	ToxA
Haiphong	18	–	–	–	5 pos
Thaibinh	06	–	–	–	6 pos
Hanoi	10	–	–	–	0 pos
	Total: 44	–	–	–	11 pos

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

	STT	Địa điểm	Kết quả MPN/ml		
			Tháng 9	Tháng 10	Tháng11
Hải Phòng (18 mẫu)	1	Hồ Tam Bạc	0	0	0
	2	Hồ Sen	0	0	36/toxR (+)
	3	Hồ An Biên	0	930/toxR (+)	0
	4	Mương Hòa Nghĩa		230/toxR (+)	0
	5	Mương Hợp Đức		36/toxR (+)	0
	6	Mương bãi rác			0
	7	Đầm Phả Lễ 1			0
	8	Đầm Phả Lễ 2			0
	9	Mương Lập Lễ 1			62
	10	Mương Lập Lễ 2			0
Hà nội (10 mẫu)	11	Sông Kim Ngưu Cầu Lạc Trung		0	230
	12	Sông Kim Ngưu Tam Trinh		0	0
	13	Hồ Thanh Nhàn		0	0
	14	Hồ Yên Sở Hoàng Mai		750/toxR (+)	11.000/toxR (+)
	15	Sông Tô Lịch ngã Tư sở		0	430/toxR (+)
	16	Hồ Linh Đàm		200/toxR (+)	0
	17	Sông Tô Lịch Đại Kim		0	0
	18	Cầu Bươu Hà Đông		0	0
	19	Cầu Sông Nhuệ		430/toxR (+)	0
	20	Ao rau muống		0	0
Thái Bình (6 mẫu)	21	Sông bệnh viện huyện Tiền Hải		0	
	22	Sông gần chỗ cất vó Tây Tiến		0	
	23	Sông gần PTTH Nam Tiền		0	

		Hải			
	24	Sông 4 cống Nam Hưng		0	
	25	Sông bãi rác Nam Hưng		0	
	26	Sông cống ông tiêu		0	
Tổng số mẫu: 44			**3**	**21**	**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 36 up to 11.000 MPN/ml.

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

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

3.5. Result of direct fluorescent antibody (DFA) assay:
Vibrio cholerae O1 and O139 were not detected from all samples.

IV.Conclusion:

- One hundred of *V. cholerae* O1 isolates collected from different provinces during period 2007 to 2009 exhibited very similar, although not entirely identical, PFGE banding patterns which suggest that they are clonal, which includes isolates of human and enviromental origin.
- MLVA revealed 25 subtypes of the isolates during the period of 2007-2009. Cholera in 2007-2009 seems to be caused by a single clone with small variations of subtypes.
- 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. *Vibrio cholerae* O1 and O139 were not detected by m-PCR and DFA methods from all samples. Elevent of NAG (non-Aglutination) were detected carried ToxR positive.

V. Recommendation:

- A combination of PFGE and MLVA analysis may yield more information about the clonality of bacterial pathogens.
- MLVA can be useful for differentiating *V. cholerae* strains that would be indistinguishable by other techniques
- The water samples from enviroment need to be collect every month during a year for *V. cholerae* ecology research.

Research 4: Establishment of laboratory diagnosis for leptospirosis and investigation of prevalence of leptospirosis among patients with fever of unknown origin in northern area of Vietnam.

Background:

Human leptospirosis is a common cause of undifferentiated febrile illness and an important health problem in Asia. Vietnam has been considered endemic for leptospirosis but information on the prevalence of leptospirosis in the country is lacking except Mekong Delta region. 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 order to improve laboratory capacity for detection of leptospira, in the present study, we apply 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. In addition, we also investigate the proportion of leptospirosis among patients with fever of unknown origin and initially identify *leptospira* in the animals and contaminated environmental samples by a *flaB*-nested PCR.

Methods:

We carried out anti-leptospiral antibody detection from serum samples which were collected from patients with fever of unknown origin at Bach Mai Hospital, Thanh Hoa general hospital. The serological method employed in this study was a whole cell-based IgM ELISA. In addition to the established method, we employed a recombinant protein (LigA)-based IgM ELISA, which we have developed recently, and evaluated the usefulness of the IgM ELISA. We also detected *Leptospira* DNA from the same serum samples and

urine by conventional PCR (*flaB*-nested PCR). Regarding DNA detection, we also evaluated a loop-mediated isothermal amplification (LAMP) method for the detection of *Leptospira* DNA directly (no DNA purification step) from urine samples. For this purpose, we collected urine samples from acute febrile patients at the hospitals and from potential reservoir animals at fields. We also tried to isolate leptospires from feral animals.

Results:

We have got 355 serum samples from unknown fever patients in BachMai and ThanhHoa general hospitals. Among them, fifty patients were obtained in Thanh Hoa. Concurrently, 100 urine samples, kidney tissues and 70 soil contaminated with animals urine have been collected from animals and environment in Thanh Hoa province.

All blood samples were tested by ELISA with whole-cell based IgM and a recombinant protein (LigA)-based IgM and showed that 36 serum samples were positive by both ELISAs while 5 samples were positive by ELISA with whole-cell based IgM, only. Thirty eight patients of 352 (10.2%) presented acute leptospirosis infection with a positive ELISA results (Fig.1). Among them, 25 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).

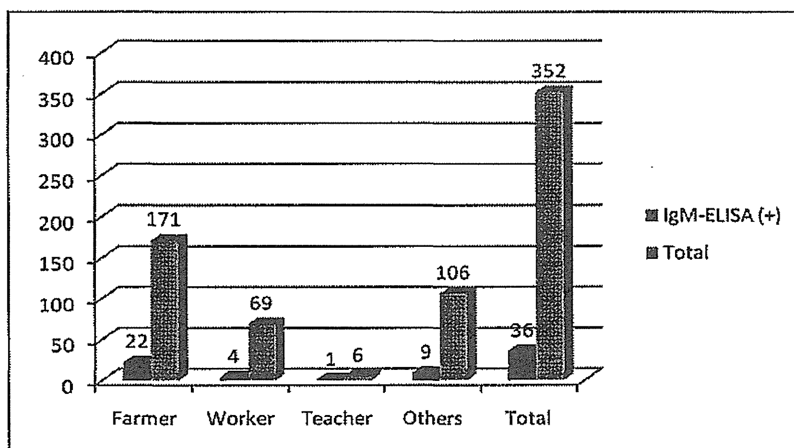


Fig.1. IgM-ELISA results from serum samples of patients

DNA has been extracted from the serum, urine, tissue samples and analyzed by nested PCR for the specific *leptospira* genes. Eight patients were presented *leptospira* DNA in serum/urine samples and eleven animal kidney tissue samples were aslo carried out *flaB* gene (fig.2).

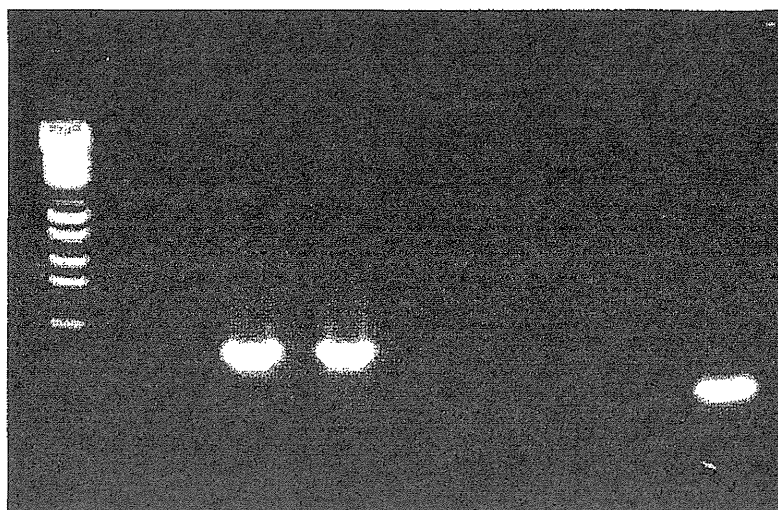


Fig.2. A nested –PCR for detection of *flaB* gene
Line 1: Maker
Line 2: sample No.1
Line 3: sample No. 2
Line 4: sample No.4
Line 5: sample No. 5
Line 6: Negative control
Line 7: Recombinant positive control
(400bp)

During the study period, we cultured 65 tissues kidney samples from dog, pig 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.

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. ELISA can be useful for detection of antibodies against leptospries, especially in acute period of disease. 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 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 10.2% of ELISA positive from suspected patients in BachMai and Thanh Hoa general hospitals. The positive results of ELISA using recombinant protein-based and whole cell-based are 41/352 and 36/352, 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. The ELISA results also showed the highest positive percentage of farmer and worker in the meat market (72.2%) to compare with other groups.

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 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. 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.

In this time, we have also applied LAMP as a rapid method for detection of *leptospira* infection. This method was performed well in NIHE laboratory. In the next step, the method should be evaluated in the field in order to rapidly detection of pathogen.

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 result showed ten patients were positive. These samples were collected from the patients with very high ELISA titer. From animal kidney tissue samples, we obtained eight positive by nested PCR but all soil samples were negative. However, our result showed that leptospirosis is presented in both human and animal in Northern Vietnam. In the next step, all positive samples will be analyzed by other molecular methods to find out a link between human, animal.

Conclusion:

The laboratory diagnosis for detection of leptospirosis has been established in Vietnam such as ELISA, culture, LAMP, nested-PCR. However, selecting an available diagnostic test is depends on the purpose of testing and on the tests or expertise available in the area. The proportion of leptospirosis in origin unknown fever patients in the study hospitals is 10.2% 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. Leptospirosis is presented in both human and animals in Northern VietNam. The further studies should be continued to find out a link between human, animals and environment sources in Vietnam.

Recommendation:

- Need to be announced to PMCs, hospital about the situation of Leptospirosis in Vietnam.
- For leptospirosis surveillance purpose, a network in combination of public health and veterinary is needed.
- There is still lacking a linkage study between human, animals and environment sources in Vietnam

Research 5: The basic and clinical study on Histoplasmosis in Vietnam

Background:

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.

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. 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.

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 are to apply a histoplasma specific PCR and serological diagnostic test in order to help rapid diagnsosis of this infectious disease and to identify the proportion of Histoplasmosis among patients with lung infection and detect *Histoplasma* in the environmental samples in order to investigate the transmission route of *H.capsultum* infection in Vietnam. Finally, making basic and clinical study network system among Asian countries is our goal.

Methods:

Study design and sample collection

This study was carried out at Infectious Department, BachMai hospital, 103 Military hospital, NIHE and some caves (Yen Bai...) and public areas in the North of Vietnam. The clinical samples include skin scrapings, sputum and bronchial washings, cerebrospinal fluid, pleural fluid, blood from suspected patients will be collected at the study hospitals. On the other hand, the environmental samples: soil contaminated with bat, bird or chicken droppings inside the caves and public areas will be collected by NIHE researchers. All samples will be transported in a safety/cool box to NIHE and stored at -80^{0}C until analyzed.

Information on disease situation, history of disease, anti-fungi use, the place to collect environmental samples etc.. will be filled in a set of questionnaire by NIHE researcher.

Laboratory procedures

According to the Histoplasma standard operating procedures of National Institute of Infectious Diseases, Tokyo, Japan.

In briefly, a suspected/infected samples will be cultured on surface of BHI or Mycocel agar; streptomycin or cycloheximide medium (cultures of *H. capsulatum* represent 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 will be followed an in house-protocol of NIID.

- The molecular methods will be identified and characterized for virulence genes by using published primers.

- Blood sample will aslo be performed by serological test following the instruction of Histoplasma DxSelect™ kit.

Results:

Up to now, 206 serum samples and 105 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, 150 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 30^0C for at least one month. Among them 72 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).

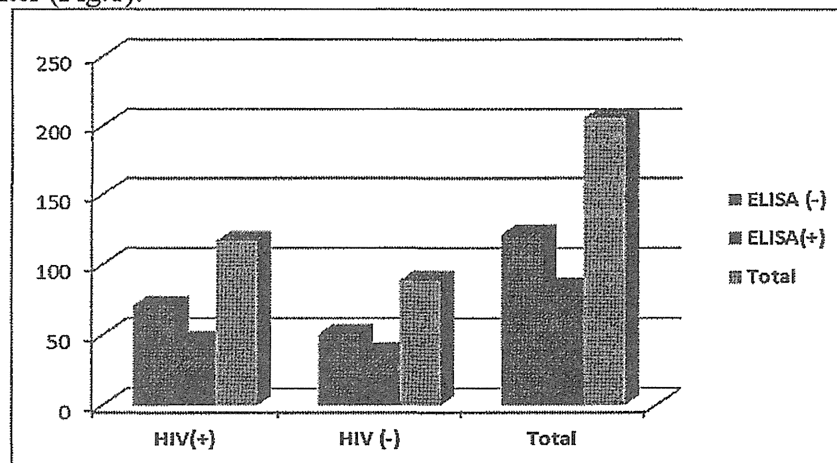


Figure 1. ELISA results of lung infected patients by HistoplasmaDxSelect™ kit

Nine clinical samples and one soil samples were positive by the nested PCR. All clinical samples positive were confirmed by sequencing. Concurrently, we have some clinical samples were positive with the first PCR using primers *Msp* 1F and *Msp* 2R encoding M antigen genes (Fig. 2).

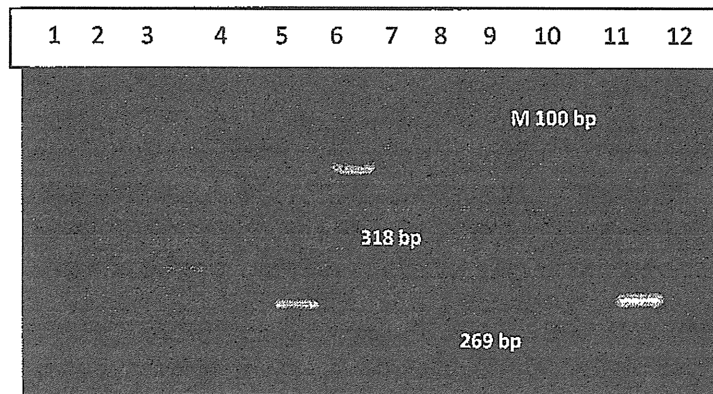


Figure 2. The first PCR results of lung infected patients

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

Discussion:

The diagnosis of histoplasmosis is based on the results of 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 considered 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, 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.

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. We have analyzed 105 clinical and 150 environmental samples by nested PCR. Nine clinical samples were positive by PCR and confirmed as *H.capsulatum* by sequencing while only one environmental positive by PCR. This samples need to be confirmed by sequencing lately. 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. 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.

Conclusion:

The 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. Due to the proportion of *Histoplasmosis* in lung infected patients is very high (40.8%). Also, *H.capsulatum* found in these patients and in environmental samples by PCR which confirmed that *H.capulatum* has been presented in Vietnam. However, the transmission route of the disease is still a challenge and need to be demonstrated in further studies.

Recommendation:

- Need to be announced to PMCs, hospital about the situation of Histoplamosis in Vietnam
- There is still lacking a linkage study between human, animals and environment sources in Vietnam on Histoplasmosis as well as fungal disease.

Research 6: The improvement of the epidemiological surveillance of Anthrax in Vietnam

Background: *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 applied a rapid and accurate detection method for *B.anthracis* from a variety of different samples and initially described the molecular characterization of *B.anthracis* isolates in Vietnam.

Methods:

Sample collection: From July, 2011 to end of August 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 and PMCs which has been created in the first period 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 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 34F2 DK strains were grown on 5% sheep blood agar and (BA) and nutrient agar (NA) 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 and centrifugated at 8000rpm/5 mins. The supernatant was used as DNA positive control for molecular typing purpose.

On the other hand, 60 fresh clinical samples were processed and cultured and/or extracted by using QIAamp® DNA mini kit (according to manufacture's instructions)

160 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.
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 (10^3) 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 PowerSoil™ DNA Isolation kit.
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.

Results:

In this study, we have identified 18 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 30 eschar and vesicular fluid samples were collected from patients. At the same time, 30 tissue samples were taken from livestock in the patient's house and market. Hundred sixty soil samples were also collected around the cage of the cow/goat and suspected patient houses in Dien Bien, HaGiang, LaiChau.

The 17JB and 34F2DK *B.anthracis* strains and clinical samples were grown well on sheep blood agar (Fig.1).

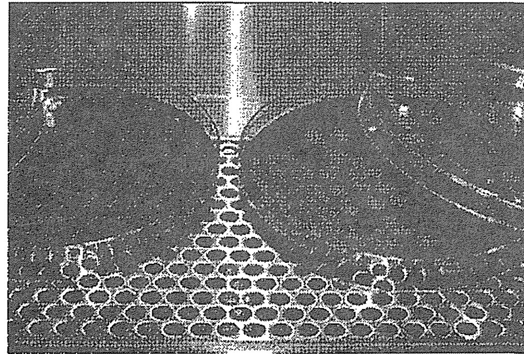


Fig. 1. *B.anthracis* colonies on blood agar plates (right site)

The clinical samples were used for both culture and DNA extraction following the standard SOP. Six 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. 2).

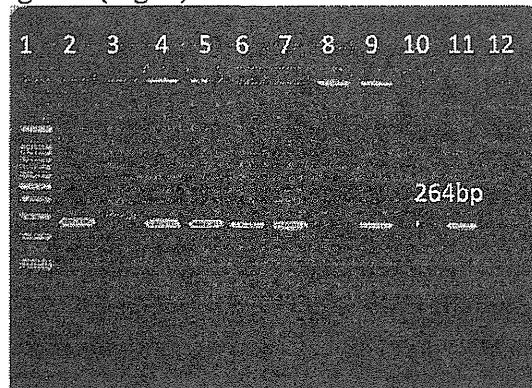


Fig.2. Identification of *B.antracis* by PCR with MO11/MO12 primers

Lane 1: molecular size marker (invitrogen)
Lane 2 – 10: clinical samples
*Lane 4& 5: same sample
Lane 11: positive control
Lane 12: negative control

The artificial infected soil sample was also positive by conventional PCR using Ba813 R1/R2 primers (showed in fig.3)

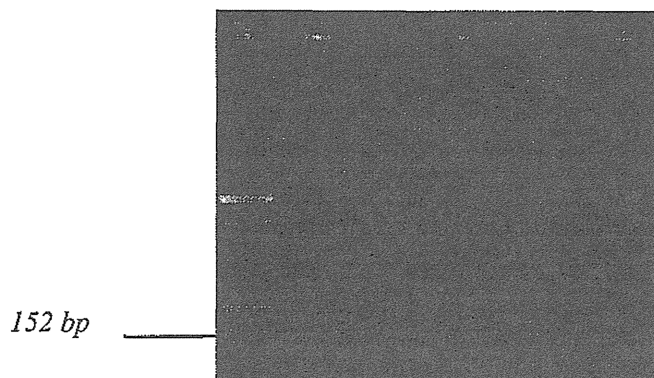


Fig.3. PCR result for detection of *B.anthracis* from artificial infected soil sample by using *Ba813* R1/R2 primers

Lane 1: molecular size marker (invitrogen)
Lane 2: 17JB
Lane 3-4: Soil samples
Lane 5: Soil + spores
Lane 6: Negative

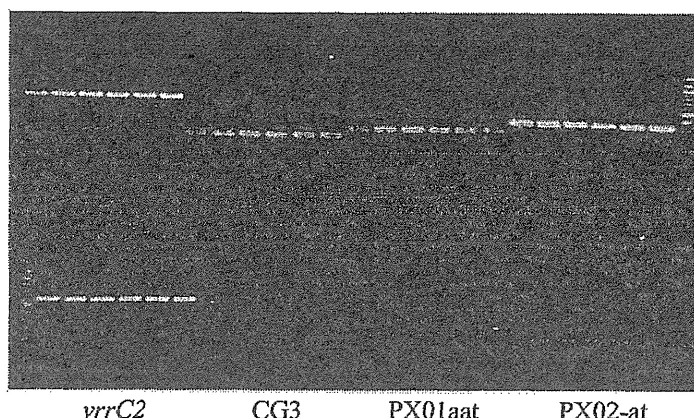
Six *B.anthracis* isolates were analyzed by MLVA-8 (Table 3). Number of tandem repeats in the loci, *vrrA, vrrB1, vrrB2, vrrC1, vrrC2* and CG3 were identical among those isolates. The isolates from LaiChau (BA1.09) and DienBien (BA2.09, BA3.09) had 7 repeats of pXO1 and pXO2 as three new *B.anthracis*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

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

Stains no.	Isolated place	Source	No. of tandem repeats by MLVA-8 determined in this study							
			vrrA	*vrrB1*	*vrrB2*	*vrrC1*	*vrrC2*	CG3	pXO1	pXO2
BA1.09	Hagiang	vesicular fluid	4	20	8	57	21	1	7	7
BA2.09	LaiChau	eschar	4	20	8	57	21	1	7	7
BA3.09	DienBien	vesicular fluid	4	20	8	57	21	1	7	7
BA4.11	DienBien	vesicular fluid	4	20	8	57	21	1	7	7
BA5.12	DienBien	eschar	4	20	8	57	21	1	7	7
BA6.12	DienBien	vesicular fluid	4	20	8	57	21	1	7	7

Fig 4. PCR reaction of each primer of marker locus

vrrC1 *vrrB2* *vrrB1* *VrrA*



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

Discussion:

Nowadays, anthrax is still considered one of the most dangerous zoonotic infectious diseases and especially, the agent has been used for biological weapon. To achieve that goal a rapid and accurate detection methods for *B.anthracis* from a variety of different products is very important. In addition, the molecular epidemiological studies with adequate monitoring and surveillance for anthrax are essential to prevent the outbreak disease and minimize its threat.

In the published studies, PCRs with the specific nucleic acid probe for *B.athracis* (no cross-reactivity with other bacterial specific) have been reported. The primers often use to amplify the target genes such as *pag, cag, lef*...that are on plasmid pXO1 and pXO2. In this study, we used the specific primers which reported by National Institute of Infectious Disease, Tokyo, Japan (table 1). As several studies reported, it is very difficult to directly detect anthrax DNA from soil samples. PCR results of the extracted DNA from 160 soil samples were negative with *B.anthracis*. Then, we made spore suspension at 10^3 concentrations from 34F2DK *B.anthracis* strain and mixed with soil sample (which was negative by culture and PCR). Results indicated that anthrax DNA from soil was detected by PCR using Ba813 R1/R2 primers.

PCR results indicated that the PCRs method could be directly detected the virulent genes of *B.anthracis* from isolate strains, clinical samples and environmental samples in Vietnam. The results also showed the advantage of PCR method because it has determined *B.anthracis* virulence genes from the clinical samples while culture result was negative. In addition, the PCR result can be analyzed by multiple-locus variable number tardem repeat to describe molecular characterization of obtained strains and compared with *B.anthracis* strains in different countries.

On the other hand, the DNA fragments can be analyzed by multiple-locus variable number tardem repeat to describe molecular characterization of obtained strains and compared with *B.anthracis* strains in different time and areas. The MLVA-8 results showed that, six *B.antracis* isolates from different time and areas are the same cluster and number or repeats in the locus (Table 3 and Fig.4). However, MLVA 8, based on only 8 locus data, and its results greatly depend on the numbers and kinds of strains used for analysis. Therefore, we used 80SNPs and determined 80 SNPs of DNA from cattle skin and tongue of DienBien and patients.
It revealed that genotype of animal and human patient strains were not