THE SECOND PHASE REPORT

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

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

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

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

5. **Research titles**

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

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

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

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

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

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

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

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

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

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

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

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

Hanoi, 15 March, 2013
Signature

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

Research 1

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

1.2 Objectives

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

1.2.2 Specific Objectives:

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

1.3 Name of Researchers

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

1.4 Affiliation

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

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

1.6 Summary

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

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

1.7 Purposes

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

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

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

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

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

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

1.8 Methods.

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

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

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

1.8.4 Specimen collection:

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

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

1.8.6 Laboratory tests to diagnose C. difficile infection:

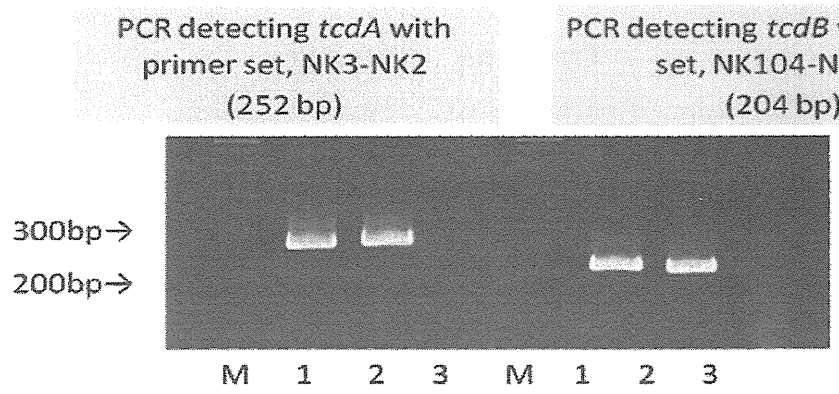
Toxin detection from stool simple by rapid immunoassay test: Toxin A and B of *C. difficile* were detected by comercial kit ImmunoCard Toxins A&B, Nissui, Japan. In comparison with the cytotoxicity assay as the gold standard for the diagnosis of *C. difficile* infection, the sensitivity, specificity,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

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

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

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

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



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

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

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

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

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

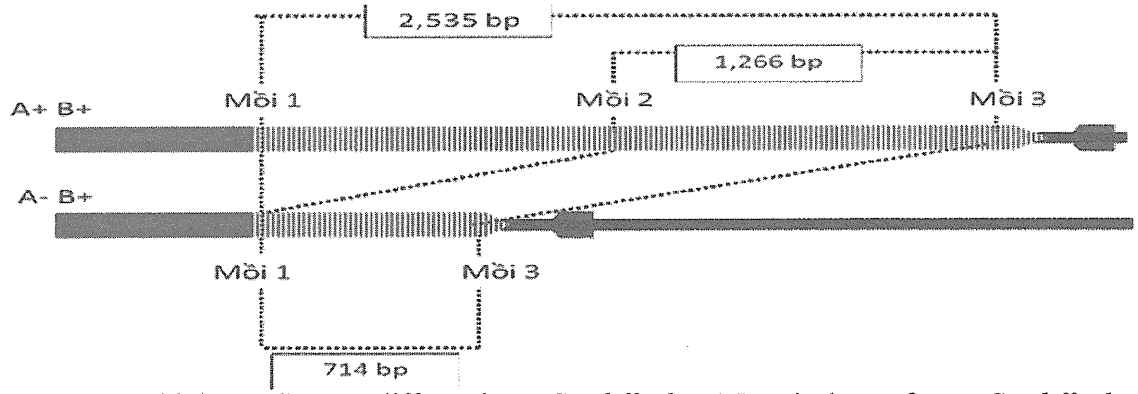


Figure2. Multiplex PCR to differentiate *C. difficile A-B+* isolates from *C. difficile A+B+*isolates.

1.9 Results

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

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

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

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

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

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

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

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

- **Toxin detection by rapid immunoassay test**

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

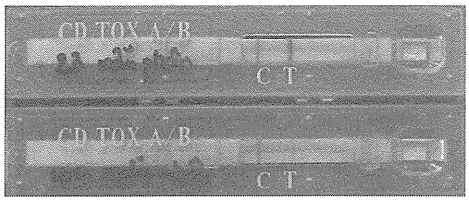


Figure 3. Toxin detection of C. difficile

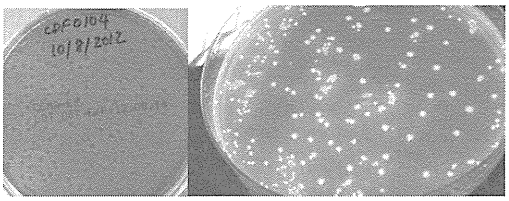


Figure 4. Clostridium difficile colonies on primary CCMA plate

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

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

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

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

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

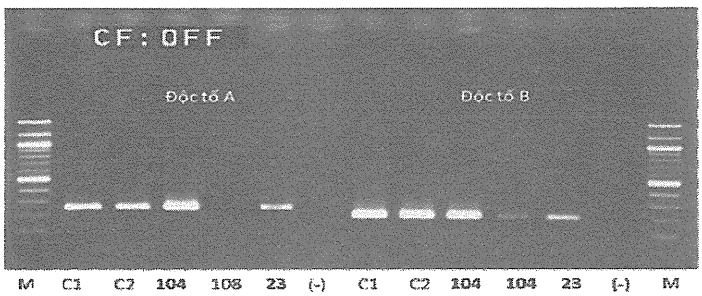


Figure 5. *PCR detecting non-repeating sequences of* toxin A gene (TcdA) and toxin B gene (TcdB) of *C. difficile. (M: standard marker; C1: positive control VPI 10463; C2: positive control G95-01; 104, 108, 23: sample codes; (-): negative control).*

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

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

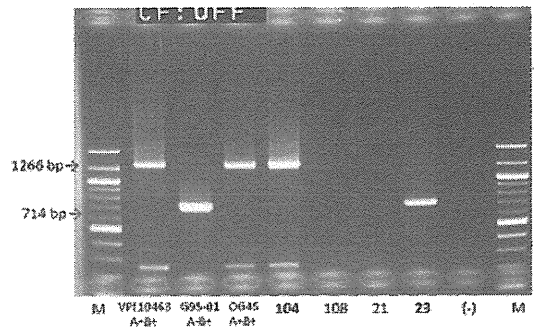


Figure 6. PCR detecting the repeating sequences of the toxin A gene (tcdA). *(M: thang chuẩn; chứng dương VPI 10463 (A+B+); chứng dương G95-01 (A-B+); chứng dương OG45 (A+B+); 104, 108, 21,23: mã số bệnh phẩm; (-): chứng âm)*

1.10 Discussion

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

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

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

1.11 Publications

1 national paper titled

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

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

1.12 Reference

1. Bartlett, J.G., *Clinical practice. Antibiotic-associated diarrhea.* N Engl J Med, 2002. **346**(5): p. 334-9.
2. Hull, M.W. and P.L. Beck, *Clostridium difficile-associated colitis.* Can Fam Physician, 2004. **50**: p. 1536-40, 1543-5.
3. Voth, D.E. and J.D. Ballard, *Clostridium difficile toxins: mechanism of action and role in disease.* Clin Microbiol Rev, 2005. **18**(2): p. 247-63.

4. Limaye, A.P., et al., *Pseudomembranous colitis caused by a toxin A(-) B(+) strain of Clostridium difficile.* J Clin Microbiol, 2000. **38**(4): p. 1696-7.
5. Toyokawa, M., et al., *Pseudomembranous colitis caused by toxin A-negative/toxin B-positive variant strain of Clostridium difficile.* J Infect Chemother, 2003. **9**(4): p. 351-4.
6. McDonald, L.C., M. Owings, and D.B. Jernigan, *Clostridium difficile infection in patients discharged from US short-stay hospitals, 1996-2003.* Emerg Infect Dis, 2006. **12**(3): p. 409-15.
7. Barbut, F., et al., *Prospective study of Clostridium difficile infections in Europe with phenotypic and genotypic characterisation of the isolates.* Clin Microbiol Infect, 2007. **13**(11): p. 1048-57.
8. Kelly, C.P., C. Pothoulakis, and J.T. LaMont, *Clostridium difficile colitis.* N Engl J Med, 1994. **330**(4): p. 257-62.
9. Akiko Ueda, M.T., Isao Nishi, Atsuko Sunada, Tomomi Sakata, and Y.I. Keigo Kimura, Seishi Asari., *Comparison of Two Immunoassays for Detection of Clostridium diffcile Toxins A and B in Stool Specimens.* Japanese Journal of Infectious Diseases, 2011. **21**(1): p. 51-58.
10. Kato, N., et al., *Identification of toxigenic Clostridium difficile by the polymerase chain reaction.* J Clin Microbiol, 1991. **29**(1): p. 33-7.
11. Kato, H., et al., *Deletions in the repeating sequences of the toxin A gene of toxin A-negative, toxin B-positive Clostridium difficile strains.* FEMS Microbiol Lett, 1999. **175**(2): p. 197-203.
12. Kim, H., et al., *Increasing prevalence of toxin A-negative, toxin B-positive isolates of Clostridium difficile in Korea: impact on laboratory diagnosis.* J Clin Microbiol, 2008. **46**(3): p. 1116-7.
13. Elliott, B., et al., *New types of toxin A-negative, toxin B-positive strains among clinical isolates of Clostridium difficile in Australia.* J Med Microbiol, 2011. **60**(Pt 8): p. 1108-11.
14. Peterson, L.R., P.J. Kelly, and H.A. Nordbrock, *Role of culture and toxin detection in laboratory testing for diagnosis of Clostridium difficile-associated diarrhea.* Eur J Clin Microbiol Infect Dis, 1996. **15**(4): p. 330-6.

1.13 Acknowledgement

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

Research 2

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

2.2 Objectives:

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

2.3 Name of Researchers

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

2.4 Affiliation

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

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

2.6 Summary:

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

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

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

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

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

2.8 Methods:

2.8.1 Study design: Cross Sectional Study

2.8.2 Study site:

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

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

2.8.4 Sample size: 794 MDR Gram-negative bacteria strains

2.8.5 Procedure:

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

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

2.9 Results

2.9.1 Detection of NDM-1 gen by PCR assay

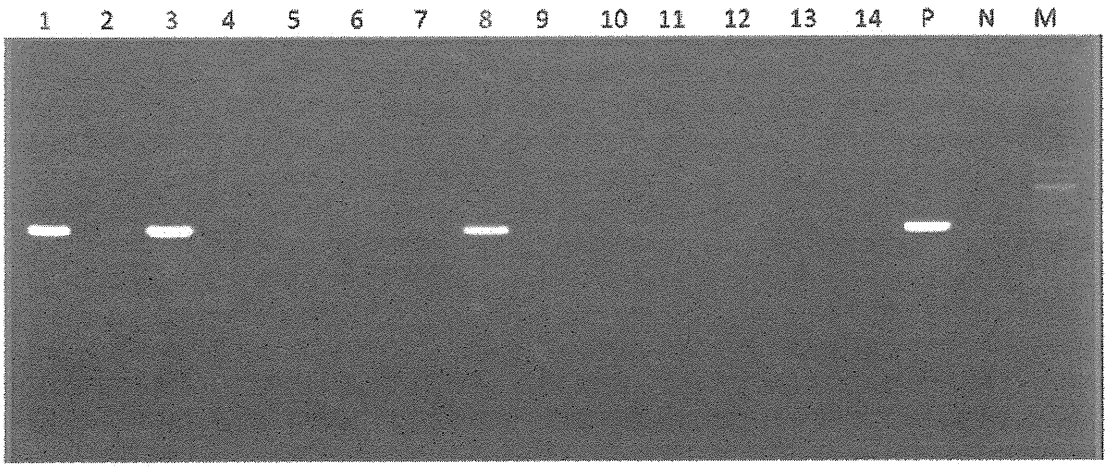


Figure 1: Amplification of NDM-1 gene: 1: *E. coli*; 3: *Proteus spp*; 8: *K. pneumoniae*; P: positive control (DNA extraction from NDM-1 *E. coli* isolated in Japan was kindly provided by Dr. Shibayama in NIID, Japan); N: negative control; M: 100bp ladder.

2.9.2 *Distribution of NDM-1 bacteria strain isolated*

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

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

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

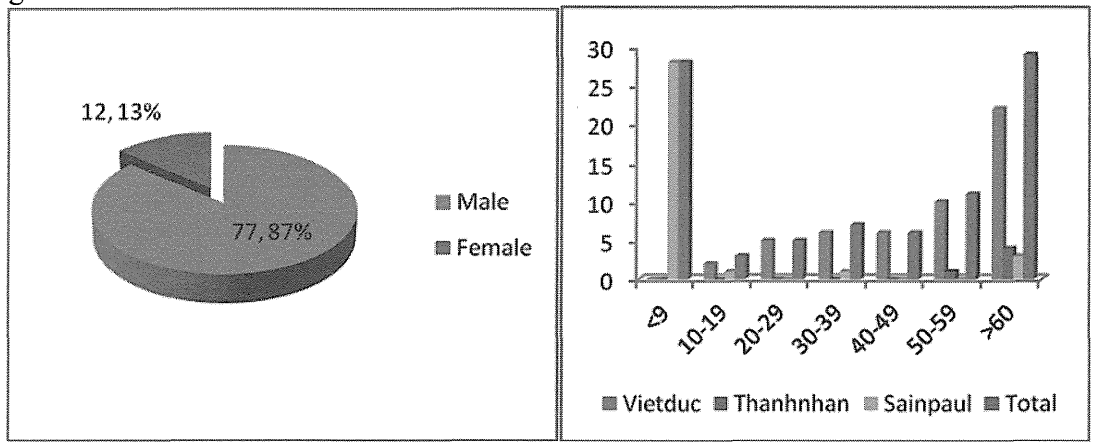


Figure 2: **Distribution of NDM-1 by sex and age groups**

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

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

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

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

Table 3: Distribution of NDM-1 strains by department

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

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

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

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

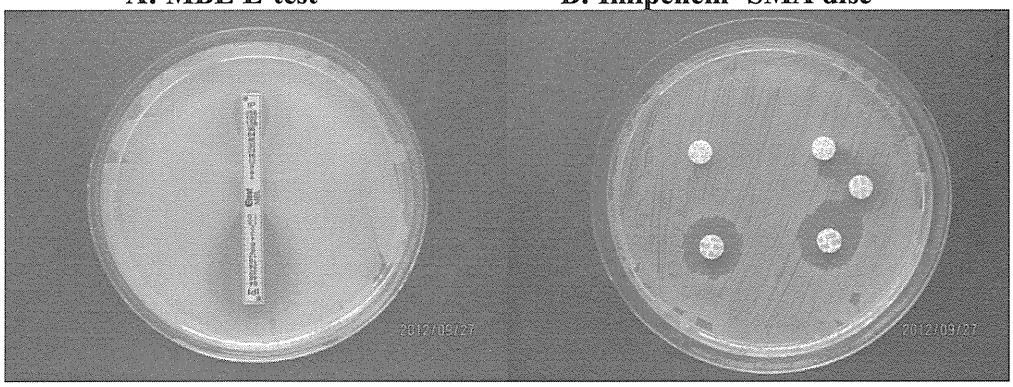


Figure 3: Metallo-beta-lactamase producing among NDM-1 bacteria isolates. A. Imipenem-SMA (Japanese kit) contained disc sodium mercapto acetic acid; B. MBL Etest (AB Bio-Merieux) contain EDTA

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

2.9.4 *Molecular characterization of NDM-1 bacterial isolates*

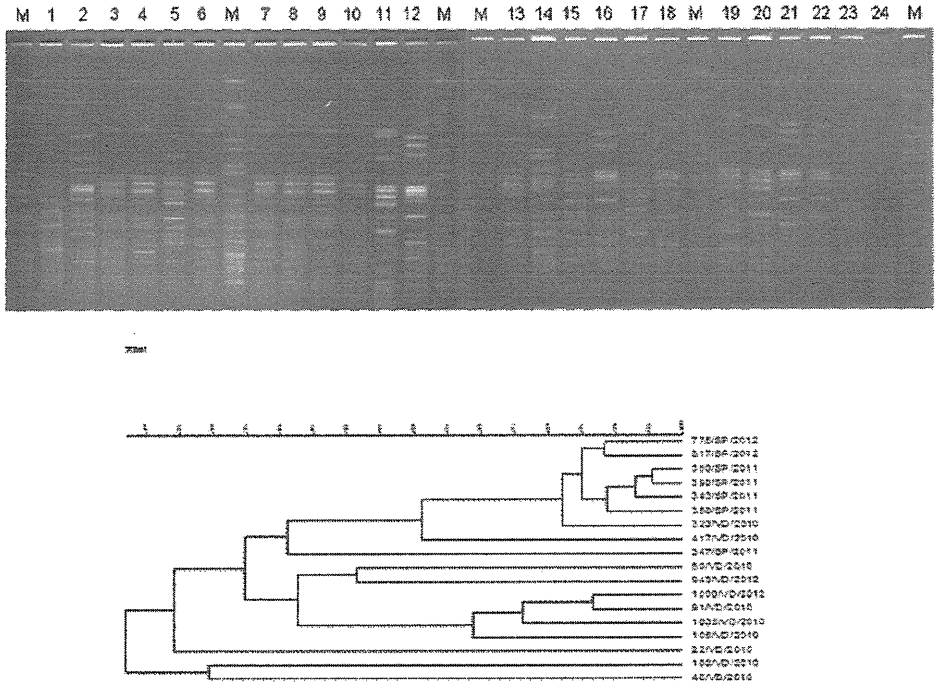
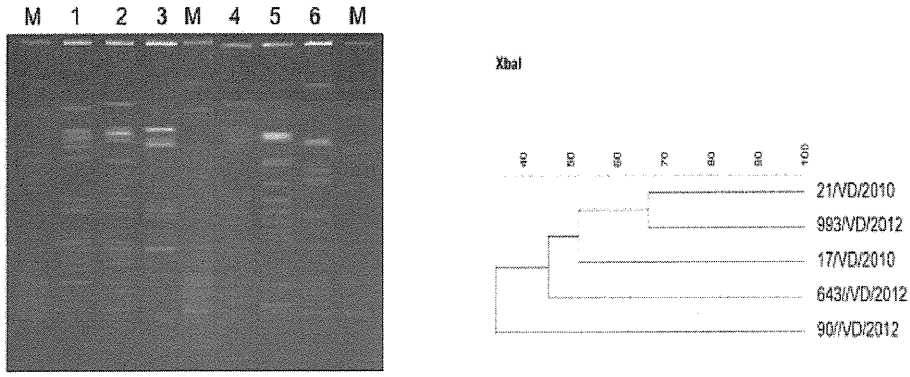


Figure 4: **PFGE profile of NDM-1-*E.coli* strains:** (1: 102/VD/2010; 2: 106/VD/2010; 3: 323/SP/2011; 4: 343/SP/2011; 5: 347/SP/2011; 6: 350/SP/2011; 7: 358/SP/2011; 8: 390/SP/2011; 9: 775/SP/2012; 10:817/SP/2012; 11. 943/VD/2012; 12: 1000/VD/2012; 13:



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

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

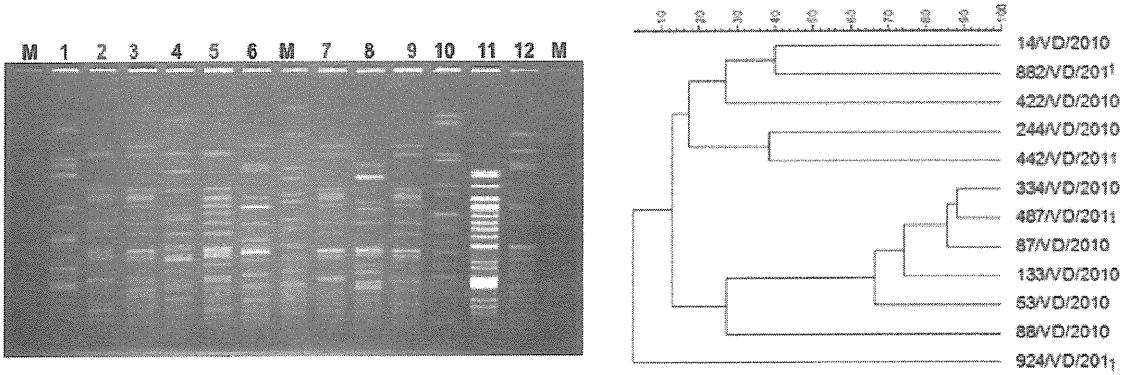


Figure 6: **PFGE profile of NDM-1-*Enterobacter* strains**: (1: 14/VD/2010; 2: 53/VD/2010; 3: 87/VD/2010; 4: 88/VD/2010; 5: 133/VD/2010; 6: 244/VD/2010; 7: 334/VD/2010; 8: 422/VD/2011; 9: 487/VD/2011; 10: 924/VD/2011; 11: 882/VD/Envi/2011; 12: 442/Saint/2011; M: *Braenderup* H9812)

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

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

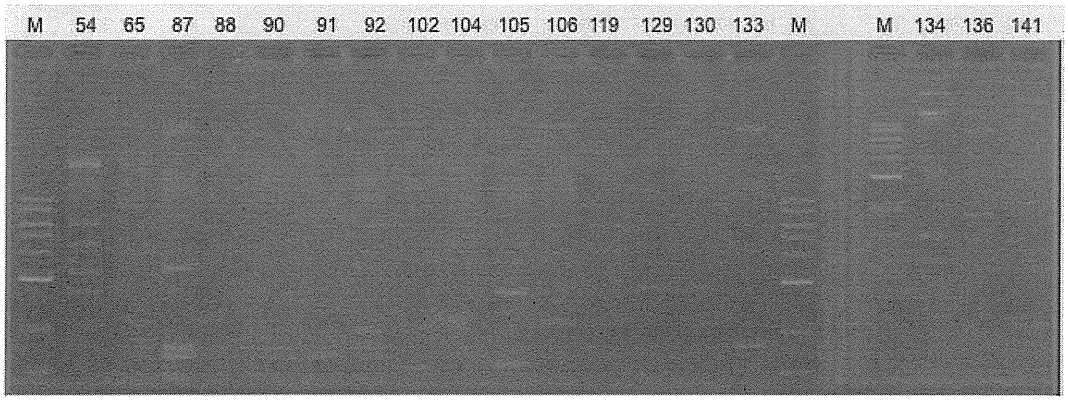


Figure 7: **The difference in plasmid numbers from a selection of NDM-1 isolates.** Many of isolates contained up to seven plasmids.

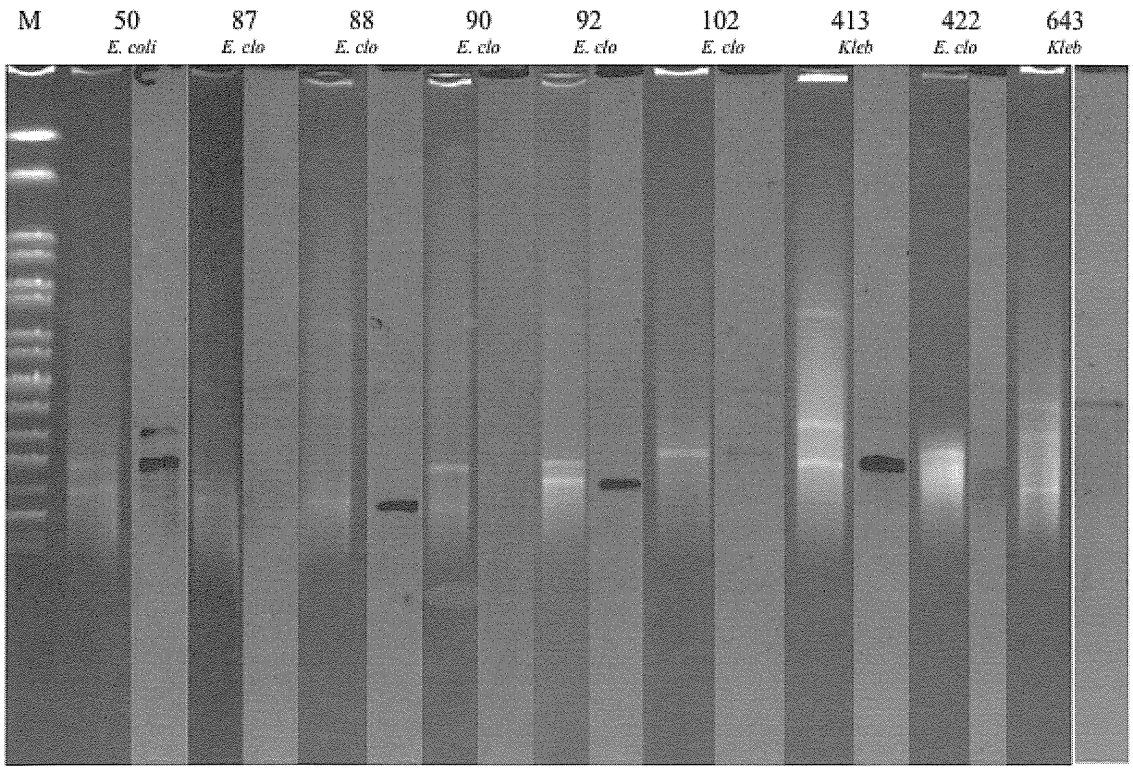


Figure 8: **Preliminary hybridization results of NDM-1 positive strains.** Pulsed-field gel of S1-treated plasmid DNA stained with ethidium bromide, M: *Braenderup* H9812-treated with *XbaI* enzyme and autoradiogram of gel probed with a NDM-1 showing individual or multiple plasmids in each strain carrying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

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

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

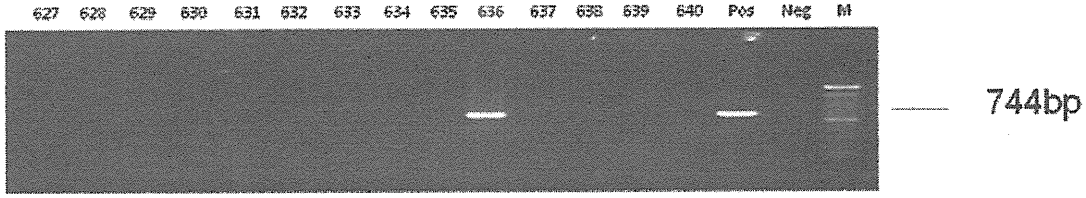


Figure 9: Identification of OXA-48 gene in bacterial isolates. 636: K. pneumoniae; P: positive control (was kindly provided by Dr. Shibayama in NIID, Japan); N: negative; M: 100bp ladder.

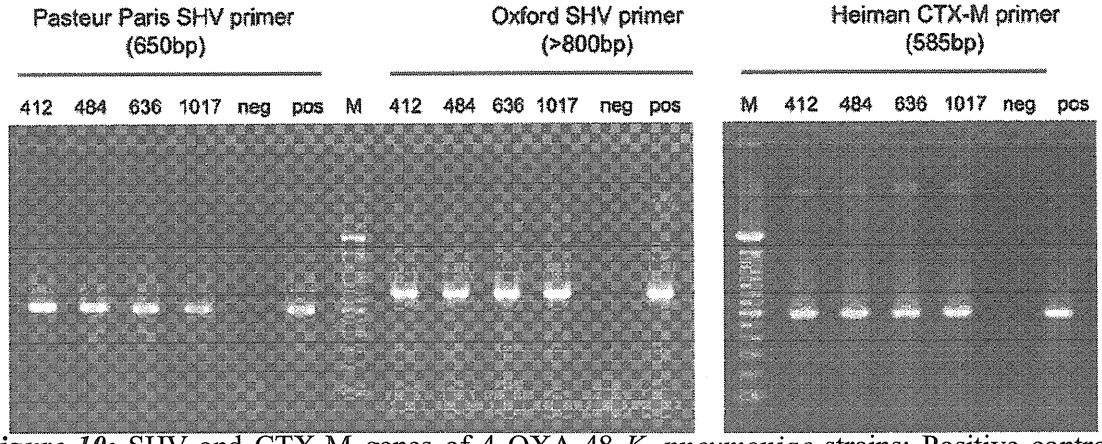


Figure 10: SHV and CTX-M genes of 4 OXA-48-*K. pneumoniae* strains; Positive control: CTX-M (*Shigella* 2009H and 10073); SHV (S. concord 07-670: TEM, SHV)

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

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

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

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

2.10 Discussion

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

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

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

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

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

2.11 Publications from this research

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

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

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

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

2.12 References

1. **Batchelor M, Hopkins K, EThrelfall EJ, Clifton-Hadley FA, Stallwood AD, Davies RH, and Liebana E (2005).** *bla*$_{CTX-M}$ Genes in Clinical Salmonella Isolates Recovered from Humans in England and Wales from 1992 to 2003 *Antimicrob Chemother*; 49,1319–22.
2. **GARP-Vietnam. (2011).** Situation analysis on antibiotic resistance use in Vietnam. *Global Antibiotic Resistance Partnership (GARP)-Vietnam*, Hanoi, Vietnam.
3. **K. K Kumarasamy, M. A Toleman, T. R Walsh, J. Bagaria, F & et al.** Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study. *Lancet* 2010. 10: 597-602.
4. **Nordmann P, Cuzon G, Naas T (2009).** The real threat of *Klebsiella pneumoniae* carbapenemase-producing bacteria. *Lancet Infect Dis*; **9:** 228–36.
5. **Nordmann P, Naas T, and Laurent Poirel L (2011).** Global Spread of Carbapenemase-producing *Enterobacteriaceae. Emer. Infect. Dis*; 17 (10): 1791-1797.
6. **Pitart C, Solé M, Roca I, Fàbrega A, Vila J, Marco F. (2011).** First Outbreak of a Plasmid-Mediated Carbapenem-Hydrolyzing OXA-48 β-Lactamase in Klebsiella pneumoniae in Spain. *Antimicrob Agents Chemother*; 55 (9): 4398–4401.
7. **Poirel P, Héritier C, Tolün V, Nordmann P (2004).** Emergence of Oxacillinase-Mediated Resistance to Imipenem in Klebsiella pneumoniae. *Antimicrob Agents Chemother*; 48 (1): 15–22.
8. **Tran Huy Hoang, Heiman Wertheim, Nguyen Binh Minh, Tran Nhu Duong, Dang Duc Anh, Tran Thi Lan Phuong, Trinh Hong Son, Hidemasa Izumiya, Makoto Ohnishi, Keigo Shibayama, Nguyen Tran Hien** (2013). Carbapenem-Resistant *Escherichia coli* and *Klebsiella pneumoniae* Strains Containing New Delhi Metallo-Beta-Lactamase Isolated from Two Patients in Vietnam. *J. Clin. Microbiol*,; 51:1 373-374.
9. **Yong D, Toleman MA, Giske CG, et al (2009).** Characterization of a new metallo-β-lactamase gene, blaNDM-1, and a novel erythromycin esterase gene carried on a unique genetic structure in *Klebsiella pneumoniae* sequence type 14 from India. *Antimicrob Agents Chemother*; **53:** 5046–54.

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Research 3

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

3.2. General objectives

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

3.3. Specific objective

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

3.4. Name of researchers:

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

3.5. Affiliation:

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

3.6. Sub-project title

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

3.7. Summary:

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

3.8. Purpose

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

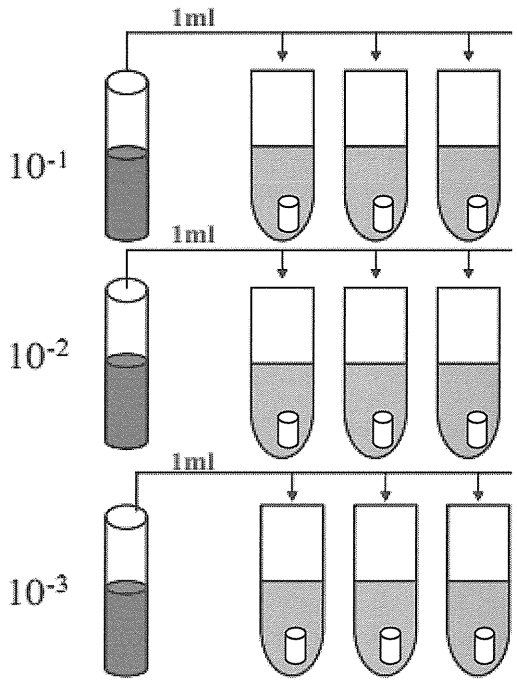
3.9. Methods

3.9.1. Most Probable Number (MPN) method

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

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

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



3.9.2. Multiplex-PCR

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

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

Table 1. Primers used in the multiplex PCR

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

3.9.3. Direct Fluorescent assay (DFA)

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

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

Principle of assay

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

Sample preparation

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

Procedure

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