

Imported Dogs as Possible Vehicles of *Vibrio Cholerae* O1 Causing Cholera Outbreaks in Northern Vietnam

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Abstract: Strains of *V. cholerae* O1 were isolated from the sewage and a pond near the first patient's house and also from domestic vegetables obtained at a neighboring market. From 24 October 2007 to 25 June 2009, 1,505 cases were confirmed positive for *V. cholerae* O1 (biotype El Tor, serotype Ogawa) in 22 cities and provinces in northern Vietnam. On May 8 and May 12, 2009, epidemic strains of *V. cholerae* O1 were isolated from dogs in slaughter houses in Hanoi and from dogs in cages in Thanh Hoa, respectively. Isolates of *V. cholerae* O1 in Laos and Thailand were found to be the same clone as those isolates from dogs, patients and environmental water samples in northern Vietnam. Although the transmission routes of cholera differed between the northern and southern provinces of Vietnam, the same clonality was observed among isolates from 2007 to 2010.

Keywords: Cholera, clonality, multiple drug resistance gene, *tetA* (class D).

INTRODUCTION

Cholera is a form of acute diarrhea caused by *Vibrio cholerae* O1 and O139. People living under poor sanitary conditions are exposed to the threat of cholera infection after consuming contaminated food and water. From 24 October 2007 to 25 June 2009, there were more than 7,000 cases suffering from acute severe, watery diarrhea in 22 cities and provinces in northern Vietnam. Of these cases, more than 1,500 were culture-positive for *V. cholerae* O1 when stool samples were tested at the Department of Bacteriology, National Institute of Hygiene and Epidemiology (NIHE), Hanoi. It is noteworthy that most of the cases occurred in northern Vietnam including Hanoi. At the beginning of the outbreak, most of the patients were among those citizens who had taken dog meat at their homes or at dog meat restaurants. There have been several reports of excretion of non-O1 *V. cholerae* (NAG vibrio) by ruminants [1, 2] and dogs [3] from hyperendemic areas and the isolation of

V. cholerae O1 from household animals from a nonendemic area [4]. However, no reports are available on the role of dogs in the maintenance and transmission of epidemic strains of *V. cholerae* O1. This communication reports the isolation of epidemic *V. cholerae* O1 strains with cholera toxin (*ctx*) gene from dogs and the clonality of epidemic strains isolated in Vietnam, Laos, Thailand, and Cambodia. Multiplex polymerase chain reaction (PCR) and pulsed field gel electrophoresis (PFGE) were used to investigate the genetic characteristics of epidemic strains of *V. cholerae* O1.

MATERIALS AND METHODS

Samples for Screening of *V. cholerae* O1

Sewage and the other Environmental Water

Samples (500 ml) were collected in clean plastic bottles and immediately transported to the laboratory at ambient temperature.

Vegetable

Vegetables (herbs and raw vegetables) were collected at a market near the patient's house and homogenized with Bag Mixer400 (Interscience, France).

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Dogs

Rectal swabs were taken from dogs in slaughter houses in Hanoi and from dog-houses in Thanh Hoa (250 km south of Hanoi) where imported dogs were kept before delivery to slaughter houses in Hanoi. Each rectal swab was kept in a test tube containing 10 ml of alkaline peptone water (pH 8.6) and transported to the laboratory at ambient temperature.

Knife, Floor, Table, Dog Meat in a Bucket in Dog Slaughter Houses in Hanoi

Samples were collected from these materials by swabbing the surface with a sterile cotton swab and kept in 10 ml of alkaline peptone water and immediately transported to the laboratory at ambient temperature.

Human Stools

Rectal swabs were taken from individuals living with a cholera patient and processed in the same way as dog rectal swabs. In hospitals, stool specimens were collected in plastic containers, and then transported to National Institute of Health and Hygiene (NIHE) without delay.

Bacteriological Examination

Water Samples

The water sample (450 ml) was mixed with 50 ml of 10x alkaline peptone water in a 1 L flask and incubated overnight at 37 °C without shaking. For enrichment, two successive subcultures were done every 6-8 hr by shaking. One ml of original culture was transferred to 9 ml of fresh alkaline peptone water. A small aliquot of the culture was plated on thiosulfate-citrate-bile salts-sucrose agar (TCBS, Eiken, Tokyo, Japan) and alkaline agar plate (nutrient agar, pH 8.6) at each step (primary, secondary and tertiary plating).

Vegetables

After homogenization, 25 ml of the sample was mixed with 225 ml of alkaline peptone water and incubated overnight at 37°C. The enrichment method was applied as in the case of water sample.

Swab Specimens

Swab specimens were incubated overnight at 37°C and two successive subcultures for enrichment were performed as above.

Suspected colonies were tested for slide agglutination with *V. cholerae* O1-polyvalent antisera (BIO-RAD, CA, USA) and with monovalent antisera (Seiken, Niigata, Japan) and were also inoculated onto Kligler iron agar, semisolid sulfide indole motility medium, Falkow's broth containing lysine, and Andrade's peptone water containing mannitol for preliminary characterization. The organisms were differentiated by biotype on the basis of their ability to cause hemolysis of sheep red blood cells [5], sensitivity to polymyxin B [6], chicken blood cell agglutination [7] and sensitivity to group IV cholera phage [8]. *Vibrio* strains were stored at -80 °C in Luria-Bertani (LB) broth supplemented with 30% glycerol.

Preparation of Template DNA

For screening of water sample for *V. cholerae* O1, DNA was extracted by the standard phenol-chloroform method from 10 ml of primary culture before enrichment.

PCR Amplification

The primers used in this study are listed in Table 1. PCR was carried out in 0.2 ml microcentrifuge tubes with 24µl of the PCR mixture containing 1µl each of forward and reverse primers (20 pM/µl) and 1µl (ca. 0.1 µg) of template DNA

Table 1. Primers Used in this Study

Primers	Sequences (5' to 3')	Amplicon Size (bp), Genes	Source or Reference; Accession No.
101-F	CCT TCG ATC CCC TAA GCA ATA C	779 (<i>toxR</i>)	[9], M21249, AE004168
837-R	AGG GTT AGC AAC GAT GCG TAA G		
CTXII	CGG GCA GAT TCT AGA CCT CCT G	564 (<i>ctxA</i>)	[10]
CTXIII	CGA TGA TCT TGG AGC ATT CCC AC		
VCO1F2-1,	GTT TCA CTG AAC AGA TGG G	192 (<i>VCO1</i>)	[11]
VCO1R2-1,	GGT CAT CTG TAA GTA CAA C		
tetA (class D) -F	TCG CTG GTA GCT TCG CTA TT	1, 327 (<i>tetA class D</i>)	In this study; AB450045.1
tetA (class D)- R	GCA ACT CTA ATG CCA CTG TG		
int1- F	ATG GCG TTA TCA GTT AGC TG	798 (<i>int</i>)	[12]; AF099172
int1- R	CTC TAT GGG CAC TGT CCA CAT TG		
Ia-F	CAA GGA AAG GTG AAC GAG TC	474 (<i>dfrA1</i>)	[13] ; X00926
Ia-R	CAC ATG CAG CCT TAT GCC AA		
dfrA1-F	CAA GGA AAG GTG AAC GAG TC	1138 (<i>dfrA1</i>)	In this study; AB 485590.1
dfrA1-R	CAC ATG CAG CCT TAT GCC AA		
intI9-F	AGC TGT TGT GAC CAG TAA CA	1, 257 (<i>intI9</i>)	In this study; AB 485590.1
intI9-R	CTT CTC AGT AAT GTC TCC AC		

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 1 min-denaturation at 94 °C, 1 min-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), stained with ethidium bromide (1 µg/ml in water), and visualized under UV light. The primers used in this study are shown in Table 1.

PFGE

PFGE was performed as previously described by Albert *et al.* [14], with some modifications. In brief, several colonies of a test strain were collected by a sterile cotton swab and resuspended in 1 ml of cell suspension buffer (CLS, 100 mM Tris, 100 mM EDTA, pH 8.0). The concentration of the cell suspension was adjusted to an optical density of 0.8-1.0 at 610 nm. An equal volume of bacterial suspension and 2% SeaKem Gold agarose (Bio-Rad, Hercules, Calif) was prepared and dispensed into a plug mold. Bacterial cells were lysed with 25 µl of proteinase K (20 mg/ml) in 5 ml of cell lysis buffer (CLB, 50 mM Tris, 50 mM EDTA, pH 8.0, 1% Sarcosyl) at 54 °C for 1 h and washed in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Restriction digestion was performed with 20 U *NotI* at 37 °C for 4 h. The agarose plug was stored in TE buffer at 4 °C. The restriction fragments were separated using the contour-clamped homogeneous electric field method on a CHEF-DRIII system (Bio-Rad) in 1% pulsed-field-certified agarose in 0.5x TBE buffer. Electrophoresis was performed at 6 V/cm at a temperature of 14 °C with the following pulse times: 2 to 10 s (13 h) and 20 to 25 s (6 h) at an angle of 120°. The gel was stained with ethidium bromide for 30 min, destained, and photographed on a UV transilluminator. The DNA size standards used were a bacteriophage lambda ladder consisting of concatemers starting at 48.5 kbp and increasing to 1,000 kbp (Bio-Rad) and a *Salmonella braenderup* strain.

Analysis of Drug-Resistance Gene Clusters

The genetic organization of the gene cluster in SXT conjugative mobile element was determined essentially by primer walking referring to the sequence of SXT^{MO10} [15]. Amplified DNA was purified before sequencing using a Qiagen Gel Extraction kit (Qiagen, Hilden, Germany) and the nucleotide sequence was determined using a cycle sequencer with an AmpliTaqFS Dye Terminator kit and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, PerkinElmer, Foster, CA, USA).

RESULTS

Recovery of *V. cholerae* O1 from Raw Vegetables and Dog Meat in a Market

Strains of *V. cholerae* O1 were isolated from raw vegetable and dog meat samples collected on 6 January 2008 at Khuong Trung market in Hanoi. Two water samples from Nhue River (Duong Noi Commune) and one sample from a pond near a patient's house collected on 4 April 2008 were found to be positive for *V. cholerae* O1. One water sample

collected from a pond in Phu Tho province on 7 April 2008 was found positive for *V. cholerae* O1.

Isolation of *V. cholerae* O1 from Dogs in Slaughter Houses in Duongnoi and Dog Houses in Thanhhoa

The delivery route of dogs imported from north-eastern provinces of Thailand is shown in Fig. (1). In Duong Noi Commune (Hanoi), 37 samples were collected from 4 slaughter houses on 8 May 2009 and 11 samples were found positive for *V. cholerae* O1. The positive samples were as follows: 1 rectal swab from a live dog, 1 rectal swab from a slaughtered dog, 2 from knives, 4 from dog-meat, 2 from water samples in the buckets to wash dog-meat, 1 from the floor surface in a slaughter house. On 13 May 2009, 54 samples were collected in dog houses in Thanh Hoa province and 3 samples were found to be positive for *V. cholerae* O1 (two rectal swabs from dogs and one bowl containing drinking water for dogs). After one week on 20 May 2009, more than 300 rectal swabs from imported dogs were examined at the Centre for Preventional Medicine, Thanhhoa. All samples were negative for *V. cholerae* O1.

Screening of the Samples from Dogs and Environmental Water Samples for *V. cholerae* O1 with Cholera Toxin (*ctxA*) Gene

Before the isolation of *V. cholerae* O1, DNA was extracted from the primary culture of water samples and rectal swabs from dogs and used as template DNA for multiplex PCR using primers for *toxR*, *ctxA* and *VCO1* genes (Fig. 2). Three water samples and 4 dog rectal swab samples showed positive for *V. cholerae* O1 with *ctxA* gene. One environmental water sample (lane 4 in Fig. 2) and a rectal swab from a dog showed the presence of non-O1 vibrios.

Clonality of Epidemic Strains Isolated between 2007 and 2009 in Vietnam

The gene *tetA* (*class D*) encoding resistance to tetracycline was first identified in *V. cholerae* O1 strain HN1 isolated in 2007 in Hanoi (accession no:AB450045.1). This new gene may work as a marker for the epidemiological study on cholera. When characterized by multiplex PCR using primers for *tetA* (*class D*), *int* and *dfrA1* genes of the SXT mobile element, the same clonality was observed among strains isolated from different sources between 2007 and 2009 in northern Vietnam (Fig. 3).

Clonality of Epidemic Strains in Laos, Thailand and Vietnam During 2007 and 2009

Four strains of *V. cholerae* O1 (2 isolates from 2007 and 2 isolates from 2008) and 12 DNA extracts of *V. cholerae* O1 (4 each year from 2007 through 2009) were provided by the Center for Laboratory and Epidemiology, Laos and by National Institute of Health, Thailand. When characterized by multiplex PCR by using primers for *dfrA1*, *tetA* (*class D*) and *intI9* genes, the same clonality was shown among isolates in Laos and Vietnam (Fig. 4). Furthermore, when characterized by single PCR using primers for *tetA* (*class D*) and *dfrA1* genes, the same clonality was observed among isolates in Thailand and Vietnam (Fig. 5). These data suggest that the epidemic clones in Laos, Vietnam and Thailand between 2007 and 2009 are the same.

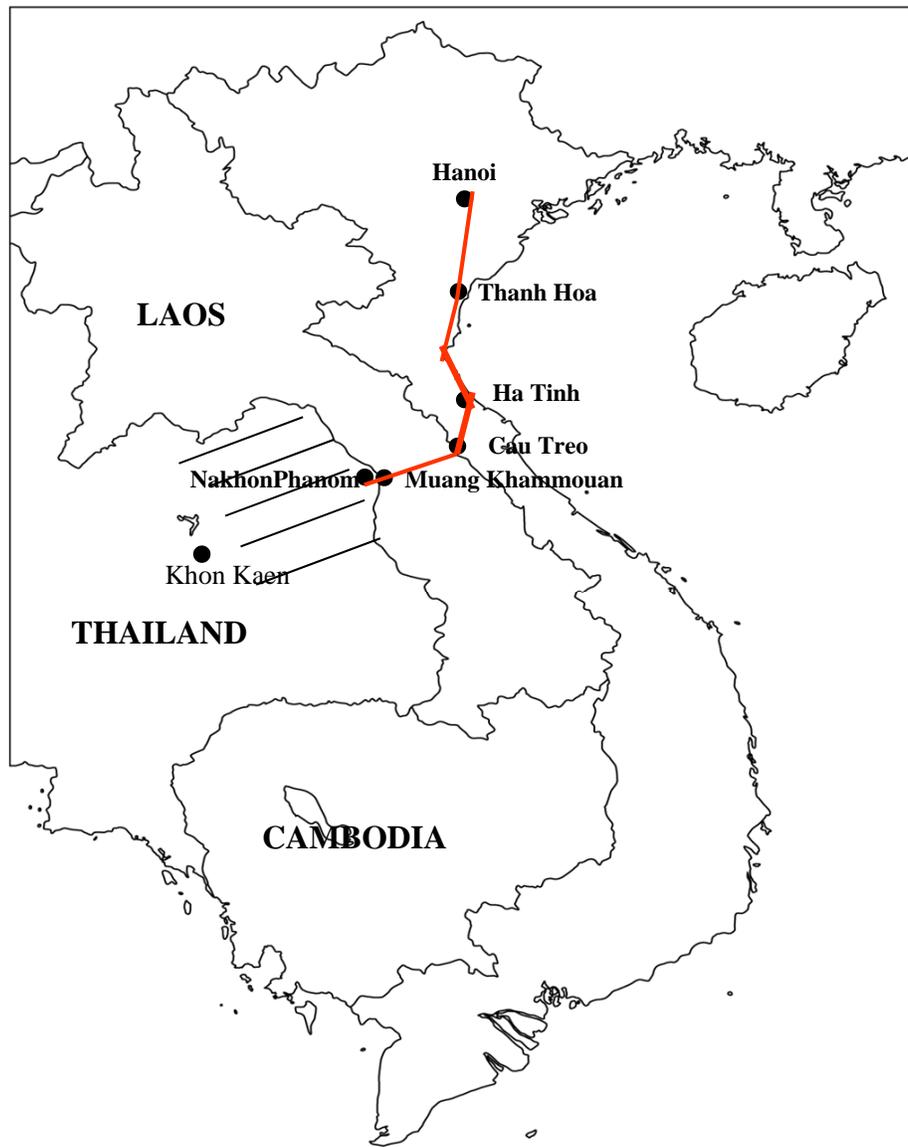


Fig. (1). The delivery route of dogs imported from north-eastern Thailand.

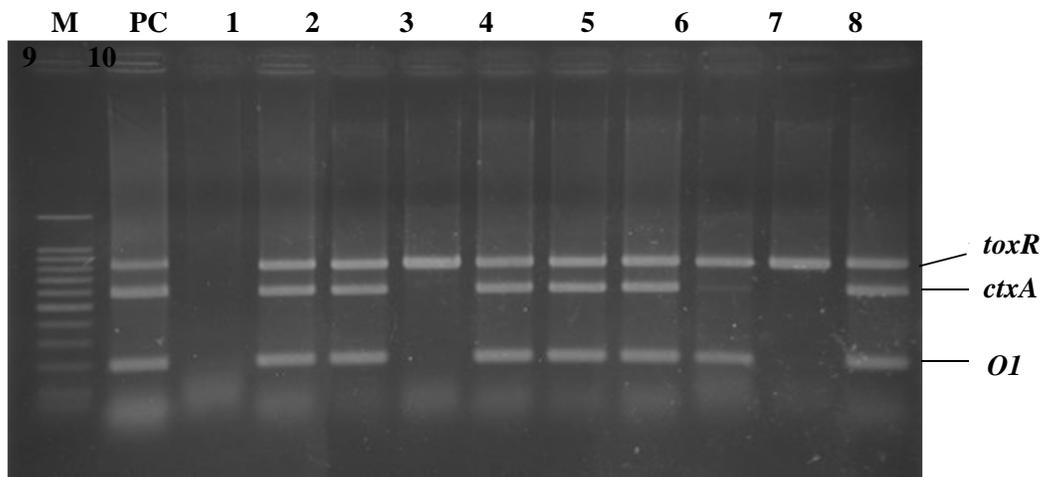


Fig. (2). Multiplex PCR to detect *toxR*, *O1*, and *ctxA* genes in water, and dog samples in Hanoi and Thanhhoa in May, 2009. Lanes M: 100 bp ladder PC: positive control (strain HN1 isolated in Hanoi in 2007): lanes 1-5: environmental water samples collected from water sources near slaughter houses in Duongnoi (Hanoi) : 6, 7: Dog-rectal swabs from Duongnoi: lanes 8-10: dog-rectal swabs from Thanhhoa. Template DNAs were extracted from primary cultures before isolation of *V. cholerae* O1.

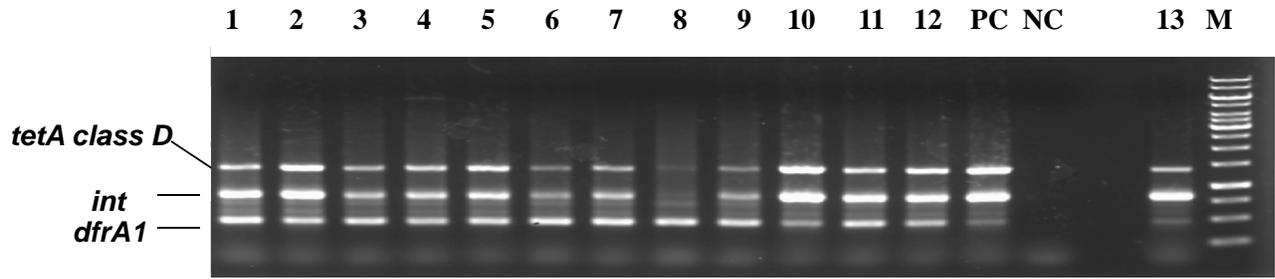


Fig. (3). Detection of *tetA* (class D), *int*, and *dfrA1* genes in *V. cholerae* O1 strains from dogs, slaughterhouses (SH) and its environment in Hanoi in May, 2009. Lane 1: floor in a slaughter house: lane 2; dog (killed), 3; dog (live with diarrhea), lane 4: knife, lane 5: dog (killed), lanes 6, 7: water to wash dog meat: lane 8: dog meat: lane 9: dog (live) lane 10: equipment in a slaughter house lane 11; dog meat, 12; Nhue River (1 km away from SH), PC: positive control (HN1), NC: negative control, 13; cholera patient's stool (Thanhhoa), M: 1k bp ladder.

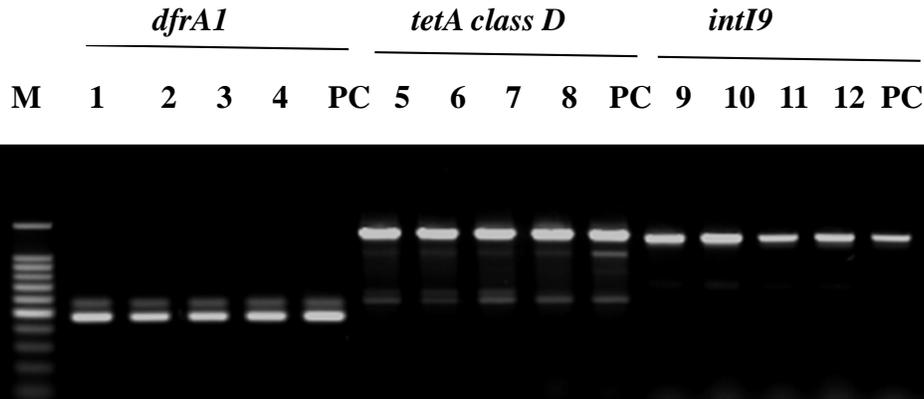


Fig. (4). Single PCR to detect antibiotic resistance genes of *V. cholerae* O1 isolates in Laos in 2007-2008. Lanes: M; 100 bp ladder, 1,2, 5,6, 9, 10; 2007- isolates, 3,4,7, 8, 11, 12; 2008-isolates, PC ; positive control (HN1)

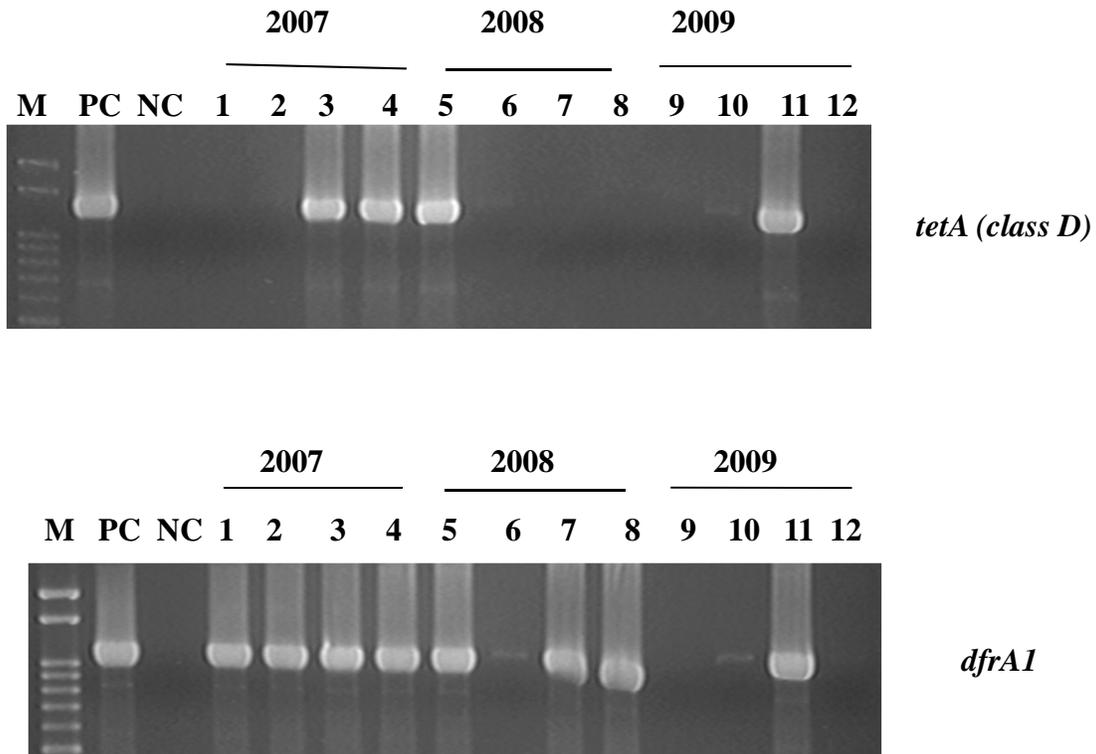


Fig. (5). Detection of *tetA* (class D) and *dfrA1* genes in Thailand isolates of *V. cholerae* O1 between 2007 and 2009. Lanes: M; 100 bp marker DNA: PC: positive control (strain HN1); lanes 1-10: Thailand isolates: VC49/50, VC54/50, VC170/50, VC440/50, VC26/51, VC52/51, VC/59/51, VC126/51, VC1/52, VC2/52, VC3/52, VC9/52, respectively.

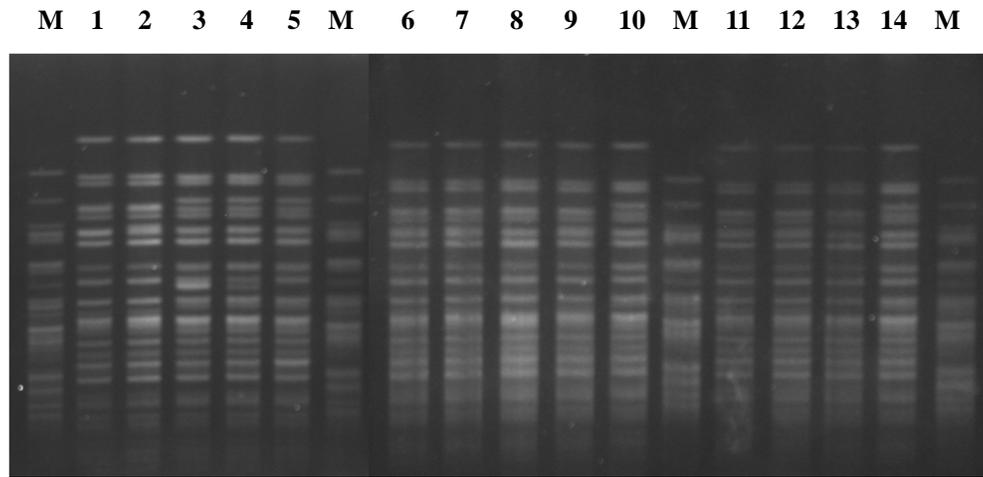


Fig. (6a). PFGE of *Not I* restriction digests of *V. cholerae* O1 isolates in Vietnam between 1995 and 2008. Lanes: M; *Salmonella braenderup* as a control, lanes 1 to 14; (strain no. isolation year); 55. 2004, 73. 2004, 55. 2007, 550. 2007, 1692. 2008, 07. 1995, 32. 2002, 272. 2003, 84. 2004, 01. 2007, 12. 2002, 307. 2003, 43. 2004, 17. 2008, respectively.

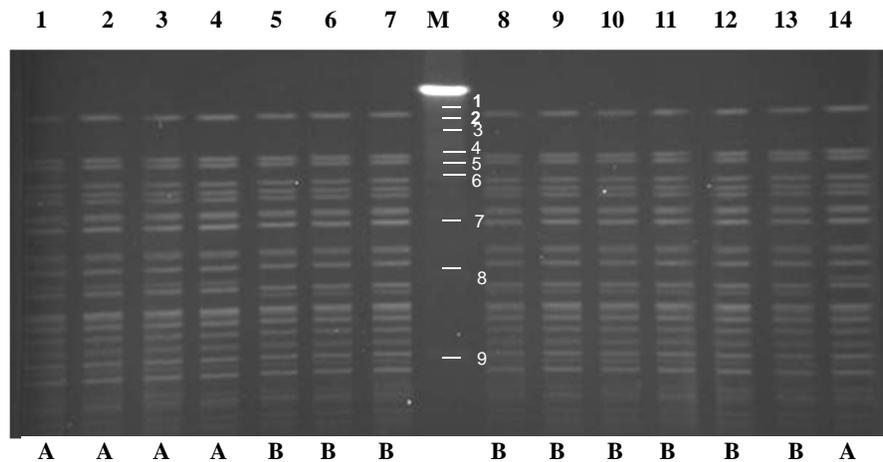


Fig. (6b). PFGE analysis of *V. cholerae* O1 isolates in 2010 in southern provinces of Vietnam. Lanes: M; marker DNA, 1 to 14; (strain no.-province); 01-AG, 115-AG, 496-BP, 43-SG, 673-AG, 21-TP, 317-TN, 232-ND, 354-BT, 672-CT, 684-CM, 586 TG, 662-BL, 781-BL. Bands 1, 2, 3, 4, 5, 6, 7, 8, and 9 are 438.5, 388.0, 339.5, 291.0, 242.5, 194.0, 145.5, 97.0, and 48.5 kb, respectively. Abbreviation of province name: AG; Angiang, TP; Ho Chi Minh City, TN; Tayninh, ND; Namdinh, BT; Bentre, CT; Cantho, CM; Camau, TG; Tiengiang, BL; Baclieu.

PFGE

One *V. cholerae* O1 isolate from 1995, 2 isolates from 2002 and 2003, 4 isolates from 2004, 3 isolates from 2007 and 2 isolates from 2008 were analyzed by PFGE after *NotI* restriction enzyme digestion. Of the 14 tested, 9 isolates from 1995 to 2004 produced similar patterns that differed from the isolates from 2007 to 2008 (Fig. 6a). Of the 13 isolates from 2010 in the southern provinces of Vietnam, two patterns, designated A and B were seen (Fig. 6b). Three samples from Angian province (lanes: 1, 2 and 5 in Fig. 6b) were obtained from Cambodian citizens who came to Angian for treatment of acute diarrhea. One strain isolated in Nam Dinh (a northern province) in 2009 produced pattern B.

Nucleotide Sequence Accession Number

The DNA sequence described in this article has been deposited in the DDBJ database under accession numbers AB450045.1 and AB485590.1.

DISCUSSION

As hypothesized earlier by Sack (3), dogs from cholera endemic areas caused the transmission of the infection in northern Vietnam. Between 1 January and 31 October 2007, there were 600 cases of cholera spread across 36 provinces in Thailand [16]. In Laos, in December 2007, cholera cases started to be reported in Sekong province with the index case being reported on 19 December. As of 19 January 2008, a total of 362 cases were registered including 3 deaths in 32 villages located in 2 districts of the province: Thateng and Lamam [17]. Acute diarrhea spread to 14 localities in Vietnam's northern and central regions, affecting 1,907 people from 23 October 2007 to 31 December 2007 [18] The 14 affected provinces included the capital city, Hanoi, and surrounding provinces. Hanoi had seen the largest number of acute diarrhea cases... At the beginning of the outbreak, no one suspected dogs as the vehicle of cholera transmission. The common food taken by patients was the shrimp paste,

because shrimp paste is always served as a sauce for dog meat.

At the end of December 2007, we collected samples from dog meat, table, floor, knife and water at slaughter houses in Duc Giang Commune, Hoai Duc District, Hanoi, but were unable to detect *V. cholerae* O1. Rectal swabs and the other swabs to collect samples were carried in test tubes with screw cap containing alkaline peptone water, it seemed there was no marked difference between rectal swab and conventional stool culture. Ponds near patients' houses were easily contaminated with the epidemic strain from human stools. Data from interviews with cholera patients conducted during the first 2 weeks of the epidemics shows that 64-83 % of the patients ate dog meat. An investigation of slaughter houses was conducted in Hanoi in May 2009 to collect rectal swabs from dogs, and water samples in buckets used for washing dog meat. We found several samples positive for *V. cholerae* O1. This is the first report of cholera transmission possibly caused by dogs imported from cholera endemic areas of neighboring countries. So far there is no report on the isolation of epidemic strain of *V. cholerae* O1 with *ctxA* gene from dogs. These dogs were imported from the north eastern province of Thailand, where cholera was endemic [19, 20].

The first isolate of *V. cholerae* O1 in Hanoi (HN1) in 2007 was analyzed genetically to characterize its properties. We found *tetA* (class D) in the conjugative mobile element (SXT element) which carries multiple drug resistance genes. The gene, *tetA* (class D) was first reported in a Spanish isolate of *Photobacterium damsela* subsp. *piscicida* by Osorio CR, *et al.* [21], this gene was never reported previously in isolates of *V. cholerae* O1. Therefore we used the *tetA* (class D) gene as a marker to discriminate strains isolated in Laos, Vietnam and Thailand together with the other genes (*dfpA1*, *int*, and *int19*). In northern Vietnam including Hanoi, fresh dog meat is prepared in Hanoi from dogs imported across the border with Laos. These imported dogs are gathered in dog houses in Thanh Hoa province and then transferred to Hanoi. Since we isolated *V. cholerae* O1 from the rectal swabs of 2 dogs from slaughter houses in Hanoi, we went to Thanh Hoa province to collect samples from dogs and from the environment around dog houses. Two rectal swabs from dogs and one drinking water sample were found to be positive for *V. cholerae* O1. The isolates of *V. cholerae* O1 from patients, imported dogs and environmental water sources collected from 2007 to 2009 were found to be the same clone as those isolates from Laos and Thailand, when judged by PCR and PFGE.

The results of PCR for Thailand isolates showed discrepancies because of the template DNA. The template DNA was prepared by the supernatant of heat-treated whole cells. Current epidemic strains in Vietnam seem to be different from those isolated before 2007. Therefore, they were probably introduced into Vietnam in 2007. Although the route of cholera transmission seemed to differ between North and South Vietnam, the same clone was seen among the isolates from 2007 to 2010. A minor difference observed in PFGE profile between isolates in 2010 from the southern provinces may be derived from some mobile elements such as SXT element and integrated filamentous phage genes. Furthermore, currently epidemic El Tor strains in Thailand

[19], Laos [22], and Vietnam [23] carry *ctx B^{Cl}* (El Tor variant). The results of multiplex PCR and PFGE [19, 22] suggested that the same epidemic clone is spread widely in Southeast Asian countries. The PFGE profile suggested that three Cambodian strains seemed to be the same clone with those isolated in Vietnam. Multilocus sequencing typing (MLST) was reported to have better discriminatory ability for typing *V. cholerae* than does pulse-field gel electrophoresis [24], and multi-virulence locus sequencing typing (MVLST) was reported to be more discriminatory than MLST [25]. Although, we used a new gene (*tetA class D*) as a specific marker for the epidemiological study on cholera, new methods such as LAMP in rectal swab could improve detection of *V. cholerae* O1.

In Vietnam, people never eat dog meat for 2 or 3 days at the beginning of every lunar month, because they believe that eating dog meat at the beginning of the month is inauspicious. This custom reflects the low incidence of acute diarrhea in the first two weeks of the lunar calendar (data not shown). Not all the dogs imported were necessarily infected with *V. cholerae* O1; some dogs in some batches were infected. A review of importation practices of animals from cholera-endemic regions may be required to prevent future transmission.

CONFLICT OF INTEREST

The authors have no conflicts of interest to report.

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