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Cholera in Lao P. D. R.: Past and Present

Satoshi Nakamura[1,7,*,*]
[1]*Department of Tropical Medicine and Malaria, National Centre for Global Health and Medicine, Tokyo*
[7]*Institut Pasteur du Laos, Ministry of Health, Vientiane*
[1]*Japan*
[7]*Lao People's Democratic Republic*

1. Introduction

Lao People's Democratic Republic (Lao PDR) is a mountainous country in Southeast Asia, situated along the Mekong and bordering China, Vietnam, Cambodia, Thailand, and Myanmar. Cholera epidemics are known to have occurred in Laos during the time of the French colonial era and the Kingdom of Laos period prior to the establishment of Lao PDR in 1975. There are public records of cholera epidemics in 1895 to 1902 of the French era (Monnais-Rousselot, 1999) and in the 1910s, 1953 and 1969 of the Kingdom era (Nakamura and Iwasa, 2008). However, after the establishment of Lao People's Democratic Republic (Lao PDR), it was presumed that there were no epidemics of cholera and, at the level of both rural and central government, cholera was not sufficiently recognized prior to 1993. Acquisition of pandemic information on cholera was severely restricted, due to the establishment of a socialist state system in Laos and its isolationist state from 1975 to the mid-1980s. Moreover, while diarrhea and/or fever outbreaks caused by unknown pathogens were common in remote areas of mountainous districts equipped with few health resources, case detection or confirmation was quite difficult because almost all the cases had ceased by the time the reports reached the central government. Since these areas had scant populations with a scattered distribution of small size villages, the roads to which they were connected were poor; thus, confirmed diagnoses of the diseases could not be made because the areas could not be accessed easily, and prudent control was not exercised with the

[*]Yutaka Midorikawa[2], Masami Nakatsu[1], Toru Watanabe[3], Rattanaphone Phethsouvanh[4], Phengta Vongphrachanh[5], Kongsap Akkhavong[6] and Paul Brey[7]
[1]*Department of Tropical Medicine and Malaria, National Centre for Global Health and Medicine, Tokyo*
[2]*Faculty of Health Sciences, Suzuka University of Medical Sciences, Mie*
[3]*Graduate School of Agriculture, Yamagata University, Yamagata*
[4]*Laboratory of Bacteriology, National Mahosot Hospital, Ministry of Health, Vientiane*
[5]*National Centre of Laboratory and Epidemiology, Ministry of Health, Vientiane*
[6]*National Institute of Public Health, Ministry of Health, Vientiane*
[7]*Institut Pasteur du Laos, Ministry of Health, Vientiane*
[1,2,3]*Japan*
[4,5,6,7]*Lao People's Democratic Republic*
*Corresponding Author

exception of the itinerary of EPI sentinels. As a result, most of the outbreaks occurred unquestioned and health measurement in these remote areas remained limited.

We will present details of the cholera situation in Lao PDR from 1993-1996, a period during which overt epidemics were reported. Hitherto, these epidemics had been little known to other countries with the exception of some documents or reports (Anonymous, 1993; Global Task Force on Cholera Control, 2008). Hence, we would like to give details of case studies and especially to clarify some characteristics of non-O1 non-139 *Vibrio cholerae* strains which have not yet been studied as a potential causative of diarrhea in the country. These results will necessarily be limited; however, they might contribute to further epidemiological study of cholera in the Southeast Asia region.

In this chapter, we refer to non-O1 non-139 *Vibrio cholerae* simply as NAG (non-agglutinable) *V. cholerae*, a former common abbreviation.

2. Cholera epidemics in Lao PDR from 1993 to the present

In Lao PDR, a cholera outbreak began in the Napo area of Hinboun district in Khammouane Province on April 7th of 1993 and spread to Boulapa (at the end of April and in June), Gnyomalath (in July), Mahaxay (in July), Takehk (in July), other parts of Hinboun (in July), Xebanphai and Nonbok districts (in August). In the same year it spread within the entire region including Nakai district, and became an epidemic of 5276 cases (including 250 deaths; a case fatality rate of 4.8%). An outbreak even occurred in the Pin district of the neighboring province of Savannakhet on May 1st of the same year, and this spread within the four districts of Xephon (in June), Nong (in June), Vilaburi (in September), and Atsaphan (in December) with a total of 1614 cases (86; 5.3%). In 1994, cases of severe diarrhea first occurred at Ta Oy village in the Toum Laan district of Saravane Province in the south, before the epidemic spread to two further districts, Lakhopheng and Vaphi, with 1111 cases (88; 11.3%) by March. A further 53 (18; 43%) cases of cholera occurred again in two areas of Nong district in Savannakhet in April of the same year. By June, the outbreak had expanded to the nine districts of Champon, Tumphon, Khamthabuli, Phin, Thapanthong, Songkhon, Xaybuli, Sonbuli, and Atsaphan with a total of 1209 cases (126; 10.4%).The number of inpatients at the provincial hospital between May 8th and June 18th was 360; this rapidly increased to 1554 (126; 8.1%) in the following three months. In the north of the country, nine cases of severe diarrhea were reported by the provincial health office of Bokeo Province in April 1994. Cases also spread to the three provinces of Oudomxay, Xaignaburi, and Luangnamtha within the same month, and two cases of cholera were reported in Luangphabang Province in May. Severe diarrhea cases spread to Xienkhouane province in the north and to Attapeu province in the south by June but cholera bacterium was not detected in patients from Attapeu Province. The official statistic report on cases of severe diarrhea including cholera in 1994 was not very precise; however, more than 5200 cases were observed during the two months from April to May.

In the year 1995, 192 cases (36; 18.9%) of serious diarrhea occurred in Attapeu between early January and the end of February. As in the previous year, the causative bacterium was not confirmed. In June and beyond, 260 cases (25; 9.6%) of cholera broke out in the four provinces of Sekon, Xaignaburi, Luangphabang, and Khammouane. From December 11-19th, a cholera outbreak with 141 cases occurred at Ban Phailom in Xaithani district, the first of its kind in Vientiane Capital (Vientiane Municipality at the time) (Nakamura, Marui,

2000), and the epidemic had spread to the whole of the country by 1996 (Midorikawa *et al*, 1996). Table 1 shows a summary of the epidemics in Lao PDR during the above period.

Year	Province	Population*	Case	Death case	Fatality rate(%)
1993	Khammouane	275,400	5521	254	4.6
	Savannakhet	674,900	1531	84	5.5
1994	Bokeo	114,900	1077	56	5.2
	Luangnamtha	115,200	1043	81	7.8
	Oudomxai	211,300	1492	92	6.2
	Luangprabang	367,200	52	4	7.7
	Special region				
	Hongsa**	-	18	3	16.7
	Xayabury	293,300	793	66	8.3
	Khammouane	275,400	611	11	1.8
	Savannakhet	674,900	2789	151	5.4
	Saravane	258,300	1315	93	7.1
	Attapeu**	87,700	688	59	8.6
1995	Luangprabang	367,200	128	4	3.1
	Xayabury	293,300	158	3	1.9
	Vientiane Mun.	531,800	141	0	0
	Sekong	64,200	610	76	12.5
	Attapeu*	87,700	530	91	17.2
1996	Phongsaly	153,000	1	0	0
	Huaphanh	247,300	83	12	14.4
	Xayabury	293,300	78	4	5.1
	Xiengkhuang	201,200	199	14	7
	Khammuane	275,400	5	0	0
	Savannakhet	674,900	4	0	0
	Sekong	64,200	75	4	5.3

* Population census 1995

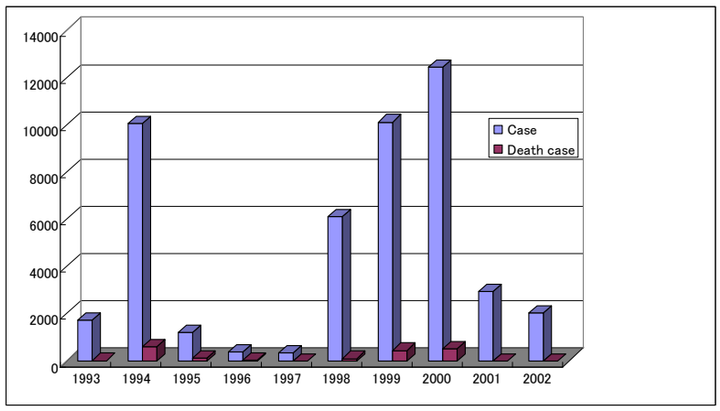
** Cholera vibrio was not confirmed

Table 1. Cholera epidemics in Lao PDR during 1993-1996.

The government of Lao PDR (GOL) responded swiftly in requesting cholera control assistance from foreign countries including NGOs through the UN Department of Humanitarian Affairs, Geneva, and received donations of 570,000 US dollars for control activities (Anonymous 1995). In addition, GOL cooperated with the WHO after 1994, a joint National Cholera Epidemic Control Committee was established in the CDD (Diarrheal Disease Control) program at the Department of Preventive Health within the Ministry of Health, GOL, and formalized control measures against cholera were advanced. Committee meetings, and training for control in the major cities took place, and more than ten examples

of active surveillance were conducted up until 1996. For example, surveillance including carrier surveys was conducted in 12 districts in 4 provinces and resulted in the identification of 306 diarrheal cases and 25 confirmed death cases from May to August, 1994. The existence of NAG *V. cholerae* in the country was demonstrated for the first time prior to 1995 through this active surveillance. In the same year, the first cholera vaccination was performed, using oral killed vaccine donated by the Vietnamese government, at several villages in Xekong and Attapeu provinces (Nakamura, 2003). Moreover, the first National Conference on Diarrhea was held in Vientiane on 14-15th December, 1994.

The cholera epidemic temporarily ceased around the end of 1996. Although cholera in the country was categorized as severe diarrhea in the diseases statistic reports of GOL in 1997 and beyond, it continued sporadically. In 1998, cases of severe diarrhea rapidly increased to 6,000, and a cholera epidemic reemerged in 2000 with more than 12,000 recognized cases, although this decreased dramatically in 2002 (Figure.1). The monthly cases of severe diarrhea including cholera from 1994 to 2000 are shown in Figure 2. According to the average of the cumulated data, diarrheal cases were most frequent between April and June with a peak in May (Figure 3), results which will require further epidemiological analysis in relation to weather conditions.



Modification of "Summarized report of surveillance on 18 symptoms/diseases classification in 2002, MOH, GOL, 2003, p.34 "

Fig. 1. Case and death case of severe diarrhea from 1993 to in Lao PDR.

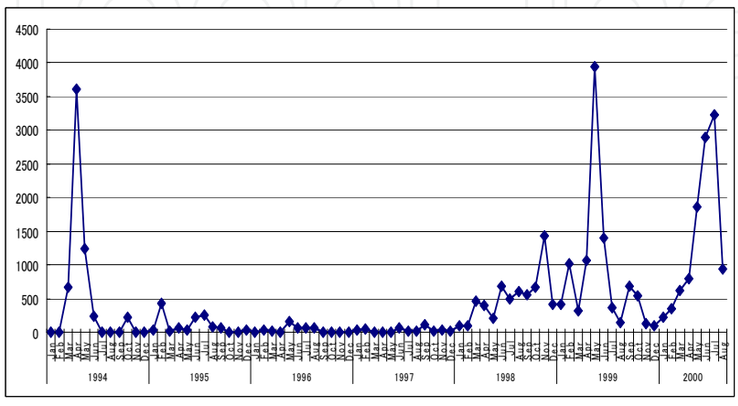


Fig. 2. Severe diarrheal cases reported by Ministry of Health, Lao PDR, Jan 1994 - Aug 2000

Although the patients' average case fatality rate exceeded 11% during the epidemic from 1993 to 1996, it henceforth fell to about 4.5% in 1998. Moreover, the rate in 2000 fell even further than previously, to 4.2%.

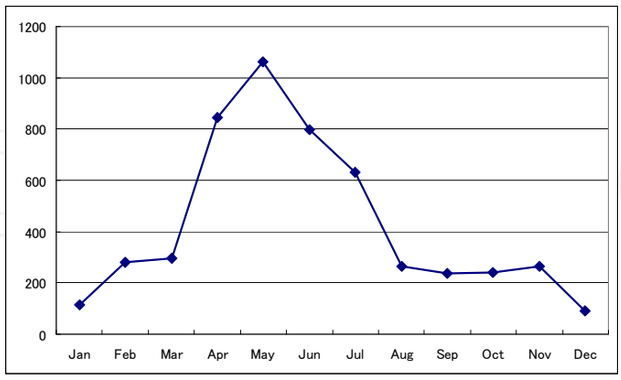


Fig. 3. Average monthly severe diarrheal cases pattern including cholera in Lao PDR from January 1994 to August 2000

In terms of epidemics in 2000-2003 and beyond, a distinct outbreak occurred in the two districts of Thateng and Lamam in Sekong Province in December 2007- January 2008 (Lenglet *et al*., 2010).

The causative vibrios collected by the active surveillance in 1993-1995 were all identified as *Vibrio cholerae* O1 serotype Ogawa and biotype El Tor at the National Institute of Hygiene and Epidemiology (NIHE) (Nakamura *et al*. 1998), and the strains contributed for further publications (Toma *et al*., 1997). Amongst these strains, antibiotic resistance was limited to Ampicillins. However, the characteristics of the cholera bacteria changed in the strains collected in 1996 and afterwards as reported in India, Vietnam and Thailand (Bag *et al*., 1998; Dalsgaard *et al*., 1999; Dalsgaard *et al*., 2000). The genomic analyses revealed that these strains were introduced with a SXT constin gene that regulates for multidrug resistancy (Iwanaga *et al*., 2004). We also speculate that the change was mediated by the presence of NAG vibrios in the country. Although a *V. cholerae* O139 epidemic occurred in Thailand from 1993 to1994 (Chongsa-nguan M. *et al*., 1993; Bodhidatta L., *et al*., 1995), we have no evidence that the strain was recovered from the country.

Even though epidemics have ceased and severe diarrheal cases have been limited to sporadic cases since 2008, it is possible that another epidemic could easily reoccur in Lao PDR because people's natural immunity to the cholera vibrios is limited to O1 serotype Ogawa, biotype El Tor and, as such, this immunity will not be very long lasting (Kabir, 2005). Moreover, in some local areas, people were indifferent about the cholera disease itself (Midorikawa *et al*., 2010). Local people also suffer from poor food hygiene related to the common custom of eating raw materials (mixed dishes known as *laap*; fish, meat, *Tao etc*.: Nakamura *et al*., 2008), as well as poor knowledge of water-food sanitation, which was confirmed by our experiences including active surveillance.

The following case studies focused on the characteristics of water-borne and food-borne aspects related to disease control in the northern part of Laos in 1994-1995.

3. Case studies of cholera in northern Lao PDR in the epidemic year of 1994

3.1 Cases in Luangnamtha Province

In early April of 1994, outbreaks of cholera occurred successively in northern Bokeo and Oudomxay provinces, and in Luangnamtha province.

No.	Village	Population	Case	Death case	Age distribution of death case	Date of onset
1	Pavi	152	4	0		10 May, 1994
2	Moklao	53	4	2	50y:f, 43y:m	12 April, 1994
3	Hatte	118	39	2	35y:f, 33y:m	19 April, 1994
4	Vath	205	51	4	4y:m, 3y:m, 1.6y:f 88y:f	1 May, 1994

No.	Locality	Age distribution >65	64-16	15-6	<5	Total
1	Moktou	3	31‡	1	0	35
2	Hattae	0	7	0	1	8
3	Nam Heng	1	5	0	0	6
4	Phouhaun	0	1	2	2	5
5	Ponhaun	0	1	1	0	2
6	Nale	1	1	0	0	2
7	Takdeth	0	0	1	0	1
8	Vath	0	1	0	0	1
9	Vienglao	0	1	0	0	1
10	Phavy	0	0	0	1	1
11	Phattana	0	0	0	1	1
12	Unknown	0	33	7	5	45
	Total	5	81	12	10	108

Table 2. The diarrhea case detection at four onset villages in Nale district on 16th May and age distribution of recorded cases treated at Nale district hospital during April to May 1994

Based on a request by the local government of Luangnamtha province, a GOL surveillance team visited Nale district as part of a national active case finding mission on May 12th-25th, 1994. The district is 86 km in distance from the capital of Luangnamtha. There was no car-road access but the Namtha River was accessible by boat at the time. The district had a population of 20,108 consisting of 113 villages in 12 communes. A wooden-made district hospital with a director and 13 medical aids and 11 nurses was located in the capital village

of Nale. The major ethnic group living in the area were *Lao Teun* (mid-mountain people). It was confirmed that 98 severe diarrhea cases including 8 death cases amongst 4 villages occurred in the district (Table 2). Also 108 cases of diarrhea inpatients were confirmed at the district hospital. Almost all the cases were of people over the age of five, as cholera epidemics are defined. The case fatality rate was confirmed as 8.2%.

Figure 4 shows that the epidemic route was from upstream to downstream along the Namtha River. The first unreported case was of a 50-year-old male who developed severe diarrhea at Moklao (or Vienglao) village on 12th April, 1994, before *Phimai Lao* (Lao New Year). The first reported case was of a 30-year-old at Hatte village, located on the opposite side of the Namtha River from Moklao, who visited relatives from Moklao and suffered from severe diarrhea for 5-6 days in the same period. Subsequently, a second case occurred in the village of a male aged over 30 who also suffered and died from diarrhea. Sixty five cases of severe diarrhea in sufferers over 10 years of age were then reported to the district health office on 19th April, 1994. Two to three days after this outbreak, an incidence of diarrhea occurred at Vath village and spread to 34 cases on 29-30th April, reported on May 1st , 1994. This village is located on the riverbank, about 3 km downstream from Hatte. A villager had returned to Vath after attending the funeral of a relative at Hatte, and subsequently contracted severe diarrhea. At the funeral, the local people had buried the body; however, the victim's clothes were washed in the Namtha River. Dwellers in the area had no toilet at the time other than excreting near the river.

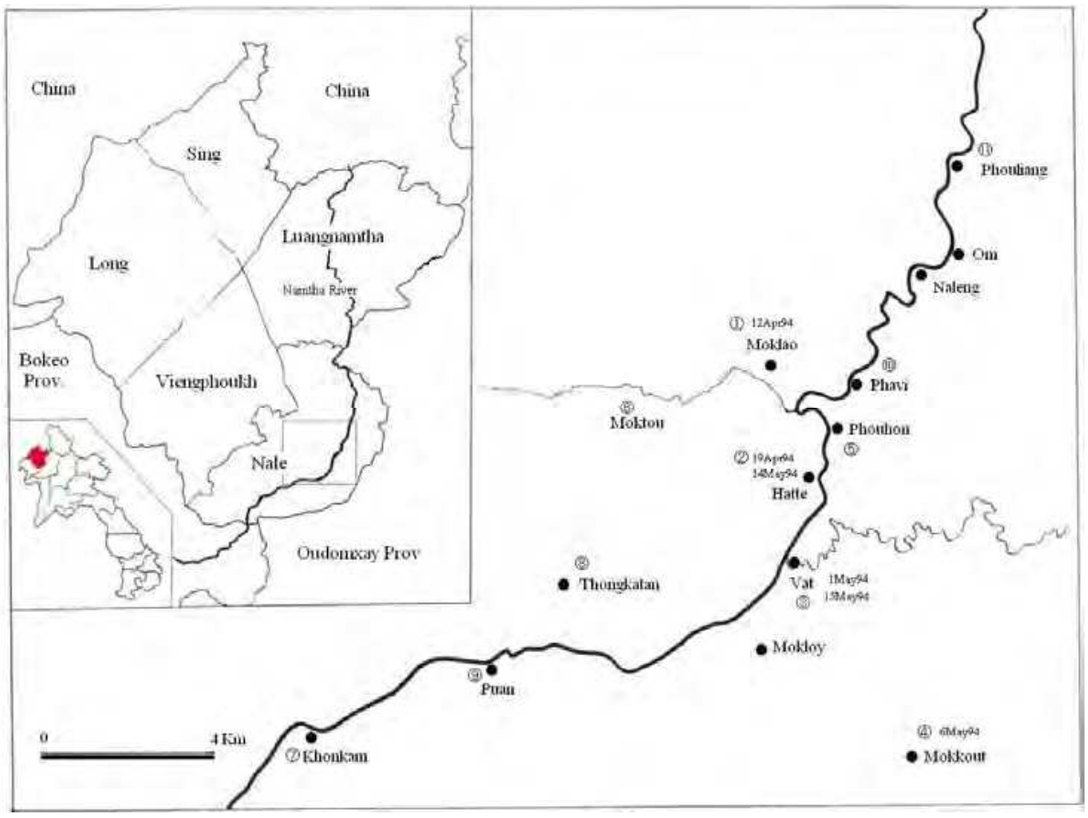


Fig. 4. Cholera transmission route in Nale district along the Nale River in Luangnamtha Province in 1994

During this mission, we also found that stools of the cholera inpatients were collected in buckets and thrown directly into the river by their family members who had stayed at the district hospital in the capital of Nale district (Picture 1). Moreover, in one case in Bokeo, the body of the deceased was also observed being thrown directly into the river by another active surveillance mission in Xaygnabouri Province in April to May 1994 (personal communication). Needless to say, cholera is a water-borne infection. It is thought that the disease was also spread through the river in a remote district of Luangnamtha Province.



Picture 1. Namtha river (left: bringing human stools to the river; right: daily life of the riverside)



Picture 2. A "spirit gate" at the entrance of a village

Interestingly, the belief that such an epidemic had been evoked as the curse of an evil spirit (*Phi* by its Lao name; man or woman) remained strong in such remote regions of Laos

(Halpern, 1963). Throughout this survey, we witnessed many charms in front of the gates of villages and the entrances of each house of villages in the district (Picture 2). People told us that the epidemic of diseases that year was caused by a female *Phi*; therefore the target was men. It was well known that rehydration therapy, such as giving oral rehydration solution to patients, is extremely effective in diminishing the number of fatalities among cholera sufferers. However, our surveillance confirmed cases of deaths in which even water was not given to the patient for fear that it would lead to even greater possession by the *Phi*. In such situations, it was thought that a control objective should have been set to improve education regarding rehydration therapy, so that skills could be gained in order to reduce cholera deaths among the local residents, even though these beliefs still remain in the present day.

3.2 Cholera expansion due to local customs and cooking food: Cases in Oudomxay Province

We now turn to cases in Oudomxay. We conducted active surveillance in the province on 6-8th June, 1994 in response to a request by the provincial government. Through the surveillance, it was reconfirmed clearly that food played a big role in the spread of cholera because it could develop in the area as food poisoning.

			Case		Age & Sex	Death	Age &	Water	
No.	Village	Population	New	Reported*	(Reported case)	case	Sex	source**	Toilet
1	Huai Ta	209	0	0		0		Strm	-
2	Kuanoy	181	0	0		0		Strm	-
3	Lak Sip	90	1	0	2, f	0		Strm	-
4	Lak Jet	NA	0	0		0		Strm	-
5	Pang Thong	>300	0	0		0		Well	-
6	Xieng Le	>200	0	0		0		Well	-
7	Nalai	370	2	0	43, m; 24, m	0		Strm	-
8	Pong Deua	397	0	0		0		Riv	-
9	Pho Keo	519	0	0		0		Well	-
10	Kon Kham	226	1	0	12, f	0		Well	-
11	Nabone	338	4	0	48, f; 20, m; 19, m; 50, m	0		Well	-
12	Houihuk	129	4	0	40, m; 30, f; 51, m; 38, f	0		Well	-
13	Pho Kham	359	2	0	40, f; 17, f	0		Well/Riv	-
14	Sam Kang	760	2	0	22, m; 20, f	1	97,f	Well/Riv	-
15	Vang Wa	329	0	2	(31, f; 65, m)	0		Riv/Strm	-
16	Pang Som	395	1	1	50, f; (38, m)	0		Fount	-
17	Namone	170	0	0		0		Strm	-
18	Vang Tang	365	0	2	(50, m; 20, m)	0		Strm	-
19	Vang Jing	230	0	1	(30, f)	0		Strm	-
20	Nangeun	287	1	0	6, m	0		TapW	+
21	Don Kham	756	0	0		0		NA	NA
22	Nakai	157	1	2	40, f; (35, m; 50, m)	0		Riv	-
23	Nam Nhone	570	0	0		0		Riv/Strm	-
24	Done Keo	714	0	0		0		Well	+
25	Oudom	471	1	0	46, f	0		Well/Fount	-
26	Saysana	718	2	0	30, f; 50, f	0		Riv	-
27	Don Saat	300	0	0		0		TapW	-
Total			22	8		1			
	* The case was already reported by the District Health Servicies								
	** Strm: Streamlet; Riv: River; Fount: Fountain; TapW: Tap water								

Table 3. The diarrhea case detection at 27 villages along the Road No. 2 in Oudomxay province during 6-8th June 1994



Picture 3. Making of pit for toilet

The surveillance team of GOL visited the four districts of Xai, Beng, Huon and Pak Beng in Oudomxay Province for active surveillance of cholera on June 6-8th, 1994. There were 27 villages, with a total population of 9500. There had been 1041 cholera cases in Oudomxay Province, including 44 death cases (Oudomxay Provincial Health Service, 7th June, 1994) and the epidemic was still continuing, as shown in the summary on Table 3. The surveillance was done only along the Route 2 road. There had been no reported severe diarrhea cases in Muan Namour and in Muan Xai districts. However, a total of 22 suspected cholera cases, including an infant and eight remainder cases, were confirmed in the surveillance. Most of the villagers in the areas had no toilet for disposing of or washing out the diarrheal stool. Therefore, a pit hole was made to dispose of the patient stool at each village (Pictue 3). One village, Oudom in Muan Beng district, suffered from a lack of drinking water from their fountain. Early in the dry season, this kind of water source was highly contaminated by faecal bacteria (Picture 4).



Picture 4. Well in a drying streamlet

The situation of cholera transmission in the province: Traditional customs for people preparing and taking meals together during ceremonies such as funerals was considered a factor in transmission in the province. For example, the first case found at Done Keo village in Huon district was on 16th April, 1994. This male case died with severe diarrhea on the same

day as its onset, and did not come into contact with the people in epidemic areas during *Phi Mai Lao*. During his funeral ceremony, many condolence callers, including relatives in Bokeo Province, visited the village. After the ceremony, his wife and his sister got severe diarrhea within a few days. After that, the diarrhea became prevalent among the villagers. We consider this kind of transmission as similar to food poisoning and it was a factor in spreading cholera from one place to another in the province. Such cases caused by cholera-contaminated foods have been reported in Africa (Lous et al., 1990). In this surveillance, we happened to observe a funeral ceremony (Picture 5, right) at Sam Kang village in Beng district on 8th June 1994. The ceremony, which involved the sacrifice of a cow, started on 6th June and participants still cooked the cow meat to eat together in front of the house of the dead patient (Picture 5, left).



Picture 5. Meal preparation (left) and the funeral of a Thai Dam villager (right).

The people of Laos, in particular, have a custom of eating raw food materials. Our observations suggested that the transmission of cholera among people in local areas depended much on cooking meals using unsafe water and on the custom of eating them using fingers at the ceremony. It is well known that a cholera bacterium can easily be disinfected by boiling, dryness, ultraviolet rays, alcohol, acid (Mata *et al*., 1994), and other means. Thus, clean food handling, sufficient cooking, and drying by sunlight for tableware, clothing, etc., are essential precautions against cholera. In particular, careful washing of hands with soap and clean water is essential before food handling. If no soap or clean water is available, hand-washing using the local alcohol *Lao-Lao* (ca.30%) or the juice of a local lime fruit (pH 4.2) called *maknao* (*Citrus aurantifolia*) is strongly recommended for disinfection of cholera vibrios. Although this recommendation was not introduced to the people, it seems that these methods are still useful to people in the local areas in Laos.

The actual transmission route of the cholera outbreak in northern provinces such as Bokeo is still quite obscure, but according to official provincial records, the first case might have occurred on 6th March 1994, before Phi Mai Lao. In Nale district, Luangnamtha Province, the first case was reported on 12th April, 1994, suggesting that quick diffusion and the spread of cholera was transmitted along the travel routes and waterways of rivers within the northern mountainous areas among Bokeo, Oudomxay and Luangnamtha provinces within April. Interestingly, there were nine cholera cases reported in Chiang Seng in

Thailand on 16th April, 1994 (information of the national cholera control committee meeting in 1994). The northern part of Laos is an important site of traffic with neighbouring countries of Thailand, Burma, China and Vietnam. Monitoring and comparing the molecular biological characteristics of the epidemic strains including NAG vibrios recovered from these areas will give useful information on controls beyond the border.

In the following section, the characteristics of the NAG vibrios recovered in Lao PDR is introduced.

4. Characteristics of NAG *V. cholerae* in Lao PDR

Some strains of NAG *V. cholerae* are a pathogen responsible for sporadic diarrhea in developing countries (WHO Weekly Epidemiological Record, 1993). The serogroup of O139 is the most widely known and studied since 1993 (Ramamurthy *et al*., 1993; Albert MJ., 1993). However, another NAG *V. cholerae* has been frequently mentioned as the causative of diarrhea in the last two decades, and is now known as enteropathogenic *V. cholerae* (Sharma, C. *et al*. 1998). To date, 200 or more *V. cholerae* serogroups have been reported, and, in particular, future epidemics of CT producing O141 strains are cause for alert (Yamai S. *et al*, 1997). Taylor, D.N. *et al* mention that NAG *V. cholerae* was frequently isolated from food and drinking water among the H'mong refugees in camps in Thailand. Despite the importance of NAG *V. cholerae* as a diarrheal cause in Lao PDR, little information on the organism was available up to now.

We report here on the serogroups and tentative results of molecular patterns of the non-O1 non-O139 strains of *V. cholerae* isolated from two areas in Lao P.D.R. during two years from 1995 to 1996.

Isolation and identification: Isolation of these Vibrio strains was carried out at the NIHE, Ministry of Health in Vientiane, Lao P.D.R. These isolates were further classified serologically with monospecific sera at the Department of Bacteriology, National Institute of Infectious Disease (NIID) in Tokyo, Japan.

PFGE: For the pulsed-field gel electrophoresis (PFGE) study, 17 strains of *V. cholerae* were classified. These included reference strains of *V. cholerae* such as the O1 classical biotype (strain 569B), O37 (African strain S7: Yamamoto *et al*., 1986) and non-typed one (strain BDD), isolated from Ban Don Daen, Khon Kaen, Thailand, respectively. *Vibrio fluvialis* O11 isolated from a diarrhea patient caused by NAG vibrio in Vientiane Capital in 1995 was also analysed as an additional reference. The genomic DNAs of the various strains were prepared in agarose plugs following the technique described by Bag P.K. *et al*. (1998). For digestion of the DNAs, 40U of *Not* I was used. PFGE of the digested plug inserts was performed by the contour-clamped homogeneous electric field method on a CHEF Mapper TM system (Bio-Rad, CA., USA) in 0.5 x TBE buffer for 40 hours 24 minutes while maintaining the temperature of the buffer at 14C. Run conditions were generated by the auto-algorithm mode of the system using a size range of 20 kb to 300 kb. A bacteriophage λ ladder (Bio-Rad) was used as the DNA molecular mass standard. The gels were stained with ethidium bromide and photographed under UV light.

PCR assay and CT production assay: A PCR-based assay was used to determine whether the *ctx*, and NAG specific heat-stable toxin (ST) were present. CT production was confirmed with RPLA commercially purchased (Denka-Seiken, Japan). The test was performed at the Department of Bacteriology, NIID, Japan.

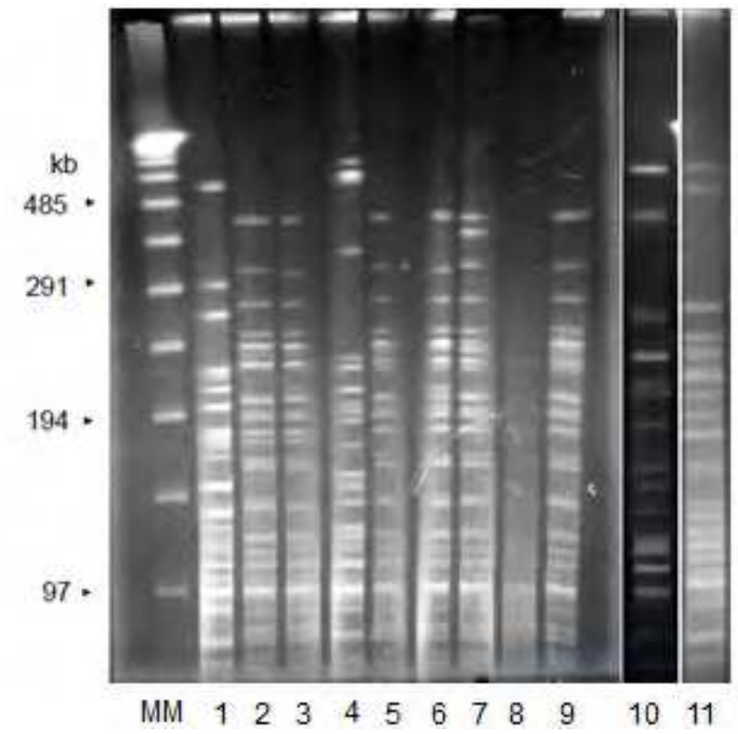
A total of 16 strains were isolated from two areas in Laos (Table 4). Among them, three strains were isolated from 533 specimens obtained by the first national cholera carrier survey at four villages in Toum Laan district, Saravane province in the southern part of the country, where a cholera epidemic occurred in May, 1994 (Midorikawa *et al*., 1996). The details of these specimens are: 468 human stools, 14 domestic animal stools, 50 drinking water samples, and 1 sewage sample at Hon Laon. These specimens were bacteriologically analyzed at NIHE in Vientiane. They included two strains of O68 and one strain of O14. Of the other 13 samples, 6 strains were isolated from 410 healthy persons who were examined after two months of the cholera epidemic at Ban Phailom in Xaithani district in Vientiane Capital on 12th December 1995, and 7 strains were isolated from 18 human diarrhea patients at a small village named Ban Lack Sao-et (meaning "21 Km village" in Laotian) located approximately 2 km from Ban Phailom in the same capital area of the country. There were variable serogroups among the isolates including O16, O21, O41, O43, and O68 at Ban Phailom, and 6 isolates of O169 and an O11 at Ban Lack Sao-et.

In the latter village, the health department of Vientiane Capital reported that 28 sporadic diarrhea cases found on 28th December 1995 were considered to be cholera. The diarrhea must have been caused by food poisoning, namely the consumption of a rice noodle call "*Khao Poun*", during a period from 16:00 to 21:00 on that day. Eleven severe cases were immediately referred to Sethathilath hospital in Vientiane. Among them, a strain of *V. fluvialis* O11 was isolated from a patient along with V. cholerae O169, and two strains of enteropathogenic *E coli* (EPEC) O159 were also co-isolated with *V. cholerae* O169 from each patient. Excluding three cases referred to but with unknown names, the age distribution was 2 children under 5 years of age, 8 between 6 to 15 years, 7 over 16 years and 1 of unknown age, respectively. Fortunately, no fatal cases were reported in this episode.

Organisms	O serogroup	Number	Source	Locality	Year
V. cholerae	O169	6	Diarrhoea stools	Vientiane	1995
V. cholerae	O11	1	Diarrhoea stool	Vientiane	1995
V. cholerae	O68	1	Normal stool	Vientiane	1995
V. cholerae	O43	1	Normal stool	Vientiane	1995
V. cholerae	O41	2	Normal stool	Vientiane	1995
V. cholerae	O21	1	Normal stool	Vientiane	1995
V. cholerae	O16	1	Normal stool	Vientiane	1995
V. cholerae	O68	2	Normal stools	Salavane	1996
V. cholerae	O14	1	Normal stool	Salavane	1996

Table 4. *Vibrio cholerae* non-O1 non-O139 strains isolated from humans in Lao P.D.R.

The representative patterns of *Not* I-digested PFGE of non-O1 non-139 *V. cholerae* strains isolated from Ban Lack Sao-et are shown in Figure 5. Of the O169 strains (lane no. 2 to 3, 5 to 7 and 9), their patterns were altogether identical except in one strain (lane 7). Other NAG Vibrio cholerae O11 (lane 4), and O21 (lane 10) were different from each other. The rest of the lanes 1, 8, 11 were V. cholera O37 (S'), V. fluvialis O11, and V. cholerae O1 (569B), respectively as reference strains.



V. cholera O11:lane 4, O14:lane12, O16:lane 11, O21:lane 10, O41:lanes 13-14,O68:lanes 15-17, O169:lanes 2-3;5-7;9, *V. cholerae* BDD (Thailand):lane18, *V. cholerae* S7 (Africa):lane 1, *V.fluvialis* O11:lane 8

Fig. 5. PFGEF profiles of NAG *Vibrio cholera* strains obtained with *Not*I enzyme.

The 16 NAG *V. cholerae* strains examined had neither the cholera toxin gene nor the heat stable toxin gene. No CT producing strains were observed among them. These results may reveal that the varied strains isolated from Ban Phailom were irrelevant to the last epidemic of *V. cholera*e O1 (Nakamura S. and Marui E., 2000). However, distinct diarrhea cases were present at Ban Lack Sao-et (21 Km village) and their major isolates were *V. cholerae* O169, which had genetic homogeneity with that of the endemic strain, suggesting that the strain was a possible endemic pathogen of diarrhea in this area. Pathogen related gene (*tcp*, *zot*, *ace*, and others) and toxin assays other than CT and the ST were not yet performed. Whether some serogroups of the enteropathogenic *V. cholerae* would cause diarrhea by a mechanism quite different from that of toxin producing *V. cholerae* O1 and O139 has not been demonstrated yet (Sharma, C. *et al*. 1998). Further analyses are necessary to clarify the relationship between genomic patterns and the pathogenicity or drug susceptibility of these *V. cholerae* O169 strains as a possible enteric pathogen in this region.

Very recently, a death case caused by the serogroup of *Vibrio cholera* O21 was reported (Phethsouvanh *et al*., 2008). This sepsis case was caused by eating a snail obtained in a swamp in the suburbs of Vientiane. For this reason, when this group was analyzed, it became clear that the serogroup has unique variation in *omp*W domain, which was named 'ompW_O21' by Nakatsu *et al*. (AB441168, GenBank, 2008). Moreover, since this strain entirely lacked the flagellum, unlike the others of O21 group included in this report, it was thought that the strain has variations in the domain of major flagellin regulation gene *fla*A (Klose KE and Mekalanos JJ, 1998). This DNA fragment related to OmpW_21 was recovered from rivers in several places

in Laos, and the fragment was also reported from tap water in the United States of America (Dross, M.C. et al.; FJ462454, GenBank, 2008). Hence, it seems that the fragment might be widely distributed over environmental water, and analysis has advanced further now.

It seems that there is a strong possibility that NAG of this country will serve as a pool of the SXT constin gene of O1 O139 *V. cholerae*. Although the place where NAG and O1 O139 *V. cholerae* in epidemics meet includes the possibility of biofilm on an animal or in nature, the most probable place is the human alimentary canal. It is necessary to advance analysis further with regard to pathogenicity about the possibility that strains of specific NAG and other *Vibrionaceae* bacteria are potential reservoirs of the drug resistance gene or of the pathogenic gene cassette as transposons.

It is reported that NAG *V. cholerae* strains are frequently separated from market foods such as meat and fish, as well as environmental water in Vientiane Capital (Nakamura *et al*., 2004, Midorikawa *et al*., 2007). Therefore, food and water source surveillance on the vibrios and their pathogen related laboratory monitoring are indispensable to health care administration in Lao PDR, which will be shown in the next section.

5. Food market and environmental water monitoring for contaminant vibrios

5.1 Observation of contamination of food by *Vibrionaceae* bacteria in the major food markets in Vientiane Capital from 2004 to 2009

In Lao PDR, the prevalence of water-borne diseases, like diarrhea, is still very high (Midorikawa *et al*., 1996; Rattanaphone *et al*, 1999). Recently, market food cross contamination by *Salmonella* and *Vibrio* species (vibrios) was reported (Nakamura *et al*.2004; Sano *et al*, 2004). Large cities such as Vientiane Capital and their surrounding areas, where people's life-style has changed rapidly, are facing the risk of environmental pollution, particularly with regard to drinking water and food. The aim of this research is to know the degree of contamination by vibrios on around 30 kinds of foods at the marketplace by monitoring food hygiene and to present an update of the possible diarrheal disease risk by using these results in the capital area. This small scale cross-sectional survey has been made by us from 1999 to the present. We presented a part of the study up to 2003 (Nakamura *et al*., 2004); also, in 2008 the analysis between some *Aeromonas* species and *V. cholerae* was not performed, and therefore we report here mainly on the results of 5 years from 2004 to 2007 and 2009.

Study site and date: The food sampling survey was conducted at two major market places in the city area in Vientiane Capital, Lao PDR. The surveys were conducted once a year, in December 2004, and in September 2005, 2006, 2007, and 2009, respectively.

Sampling of food: Objective foods of various kinds were categorized as follows: domestic animal meats (beef, water buffalo, pork, chicken, duck, and domestic fowl eggs), fresh water products (fresh water fish and shellfish), marine products (marine fish and shellfish), and others (frog and/or insect), sold at the marketplaces.

Equipment for sampling: In collection of food specimens, "Fuki-Fuki test kit" (EIKEN Kagaku, Tokyo) and "Seed-swab No.1" (EIKEN Kagaku, Tokyo) of the monitoring and transport media for food-borne bacteria were used. The surfaces of food samples were wiped with this equipment.

Isolation and identification of the bacteria: The cotton part of Fuki-Fuki test kit sample and Seed-swab or Cary-Blair kit was applied to peptone water for growth culture media, and subsequently TCBS medium was employed for selective culture of vibrios at 37C for 24 hours. Colonies on the selective medium were screened with classical *InVic* system (Phetsouvanh *et al*. 1999) and the suspected bacteria were identified with commercial identification kits such as API 20e system (bioMerieux, Tokyo, Japan). Identification of the family *Vibrionaceae* was performed using the criteria of the multiply in 0% and 7% saline broths for halophilic species, string test using 0.5% solution of sodium deoxycholate in saline added (Keast & Riley, 1997), and O129 disk susceptibility for differentiate of genus *Vibrio* and *Aeromonas*. O-antigen serotype of identified *V. cholerae* strains were determined further using diagnostic anti-O1 and anti-O139 antisera (DENKA Seiken, Tokyo).

Observation of bacterial contamination in the market foods in 2004-2009: Excepting the results of the year 2008, a total of 166 food items were examined during six years from 2004 to 2009. Suspected food poisoning *Vibrionaceae* species contamination was widely confirmed on the surface of food of animal origin (Table 5). *Aeromonas* spp including *Ae. hydrophila*, *Ae. sobria* detected from 68 items was the most prevalent except among marine fish. NAG *V. cholerae* strains confirmed in 50 food items was the next most prevalent. Among these, 26 (52%) were confirmed in the meat of domestic animals, such as cattle and pork. Moreover, contamination by the other vibrios including *V. parahaemolyticus* was commonly detected in the same category. In particular, the recovery of *V. parahaemolyticus* and other halophile vibrios from freshwater fish is uncommon in developed countries and suggests that some cross contamination reported by us is still frequent in the markets (Nakamura *et al*., 2004).

There are still insufficient regulations within food laws regarding food handling in the markets of Laos. It is still observed that the degree of cleanliness and the state of order change greatly according to each retailer's counter. Moreover, changes in collection time and sampling place also greatly influence these kinds of investigative results.

	Number of	Number of item found of Bacteria of Vibrionaceae (%)			
Food Item	items examined	non-O1, non-O139 *V. cholerae*	*V. parahaemolyticus*	Other *Vibrio* spp.	*Aeromonas* spp.
Cattle	25	7 (28) *	2 (8) *	3 (12) *	11 (44)
Buffalo	7	2 (28.5) *	0	2 (28.5) *	1 (14.2)
Pork	33	7 (21.2) *	1 (3) *	8 (24.2) *	12 (36.3)
Chicken	23	8 (34.7) *	0	9 (39.1) *	8 (34.7) *
Duck or other poultry	9	2 (22.2)	0	2 (22.2)	3 (33.3) #
Fish	51	18 (35.2) #	2 (4) *	8 (15.6) *	26 (50.9) #
Shellfish	7	4 (57.1)	1 (14.2)	1 (14.2)	4 (57.1)
Marine fish	5	1 (20) #	0	1 (20) #	0
Marine shellfish	4	1 (25) #	2 (50)	2 (50) #	3 (75)
Others (Insect & frog)	2	0	0	2 (100)	0
Total	166	50 (30.1)	8 (4.8)	38 (22.8)	68 (40.9)
* Common contamination					
# Uncommon contamination					

Table 5. Number of food items found containing *Vibrionaceae* species in the major markets in Vientiane Capital during years from 2004 to 2007, and 2009

O antigen typing and analysis of diarrheagenic toxins of these NAG *V. cholerae* have not yet been performed; however, the possible risk of severe diarrheal outbreaks was evident, as mentioned in section 4 of this chapter.

The detection rate of the food items contaminated with bacteria of *Vibrionaceae* among small size sampled food items at two major markets in Vientiane Capital from 1999 to 2009 is

shown in Figure 6. Due to the sample size, and variability within the sampling place, a direct comparison of each set of annual data is difficult; however, the results in 2004 and afterwards are reviewed here. The detection rate of food contaminated by NAG *V. cholerae* varied widely from 0 (December 2004) to 64% (2009). However there was a tendency of decreasing rates from 57% to 19% during the years from 2005 to 2007. According to Disease Statistics of Laos in 1999-2002, many food poisoning cases occurred from April to May, and the case occurrence was lowest in September (Anonymous: statistics, 2003). Since most of our investigations were conducted in September, it is necessary to similarly investigate in April and May when food poisoning occurs frequently, and to grasp the actual conditions of *V. cholerae* contamination among the foods. In addition, a more advanced investigation should also be performed on rats, flies, and sewage which are all regarded as reservoirs in the marketplaces.

In investigations into enteropathogenic bacteria carriers among residents in the year 2005 in the suburb of Vientiane, a carrier of *V. cholerae* (1.5%) was detected among 63 healthy volunteers. In the same investigation, Nakamura *et al*., (2005) confirmed that some of the Salmonella recovered from humans and market foods in the capital city showed common DNA restriction patterns. It must be emphasized that the tendency towards frequent detection of NAG *V. cholerae* from livestock meats in the marketplaces of Vientiane will be a potential risk of diarrheal outbreak.

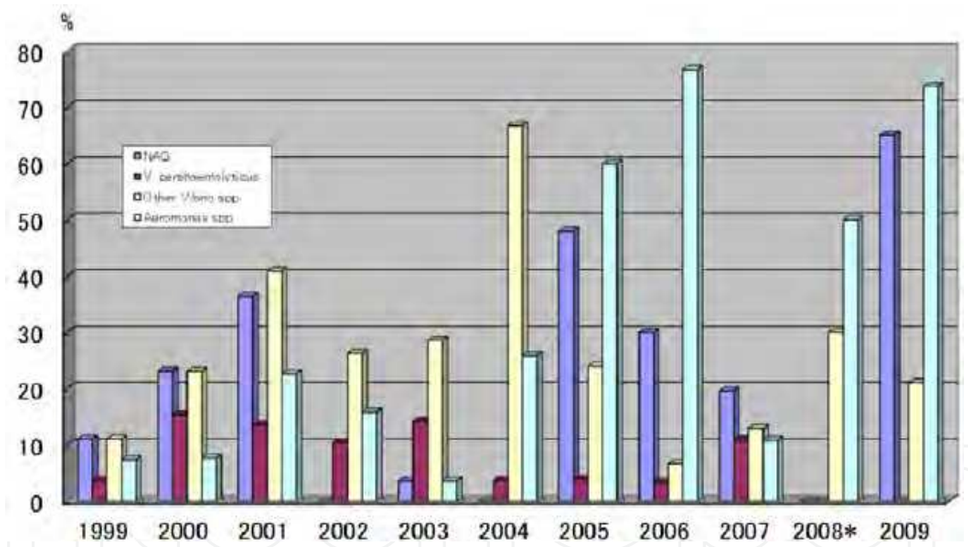


Fig. 6. Percent recovery of *Vibrionaceae* species from the foods at the two major markets in Vientiane Capital, Lao PDR. from 1999 to 2009

5.2 Environmental water monitoring on contaminant vibrios

The population density of the country is very low and it has maintained abundant water in its rivers with dense forest covering its mountainous areas. However, access to safe water is very limited among the 70% of the population living in the countryside, and risks have been pointed out about infections caused by environmental water consumption. Although the tackling of water-borne infections such as severe diarrhea among children has been an important subject for attaining MDGs of the country, there is little research in connection

with actual risk, with the exception of a few study reports. In particular, research on the actual conditions of the pathogenic organisms in the country's water cannot be found. Hence, continuous monitoring research which targets vibrios and amoeba of enteropathogenic importance was conducted to clarify distribution of these pathogenic organisms in the country.

Study area, monitoring point, sampling and the detection methods during years 2006, 2009-2011: The investigation was conducted from Vientiane to Attapeu along the catchment of the Mekong (Figure 7). Collecting river water samples of the Mekong and its branches was conducted mostly at fixed locations which cross the major national road No. 16 during the year 2006 and years 2009-2010. The water sampling along the Nam Som River and from lake water was performed in Vang Vieng district, Vientiane Province and in Khammouane Province, respectively, in 2011.

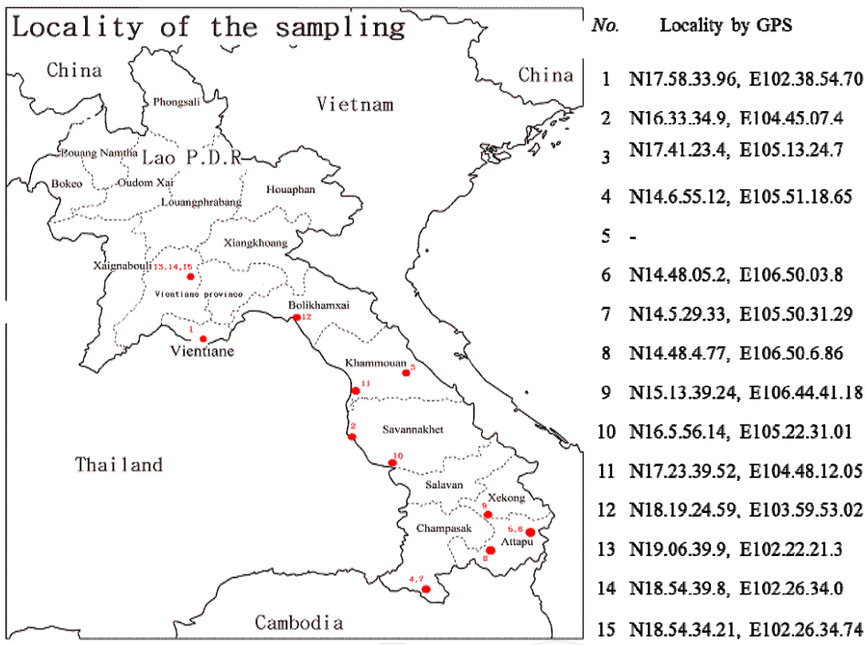


Fig. 7. Locality of the water sampling.

Water sample collection was performed with a sterile plastic container. Before collecting the sample, a conventional on-site coliform test using filter-paper (Sancoli, Tokyo, Japan) was conducted. In 2006, 20L of the raw water was condensed by DEAE to adsorb the microbes and about 1/200 of the volume was analyzed by PCR. During years 2009-2010, about 50 ml of the sample was collected to detect the target organisms by PCR. Common bacterial culture using TCBS (Eiken Kagaku, Tokyo, Japan) and DHL (Eiken Kagaku, Tokyo, Japan) media was also performed to detect vibrios and related enteropathogens as described in section II, except in 2006. Identification of bacteria was performed by API 20e system (BioMerieu, France). PCR analysis of a cultured sample using 10 ml of peptone water was also performed in 2011. All the samples were kept in cool and/or freezing conditions before analysis. PCR test for detection of the target organisms was as follows: DNA extraction from the water sample was performed on both the centrifuged pellet and the supernatant using commercial extraction kits. Target regions of PCR on *V. cholerae*, and *Entamoeba histolytica*

and *E. dispar* were ompW (Nandy *et al*. 2000, Nakatsu *et al.* 2008) and mitochondorial rDNA 18s regions with the primers newly designed by us, respectively. The PCR tests were validated using a laboratory strain of *V. cholerae* 569B and both cultured strains of *E. histolytica* and *E. dispar* were used as controls. Nested-PCRs were performed in a DNA thermal cycler with initial denaturation at 95C for 5min, followed by 35 cycles of denaturation at 95C for 30sec, annealing at 55C for 30sec, extension at 72C for 45sec and a final elongation at 72C for 7min. Commercially-based sequencing of PCR products was also obtained to confirm homology analysis using DNA databases.

Results of PCR test for *V. cholerae*, *E. histolytica* and *E. dispar*: 25 water samples were collected from 15 sampling locations. The details are shown in Table 7. Although *V. cholerae* was detected in two locations, it was detected by the second PCR. Since these did not have a ctx gene, they were judged to be NAG vibrios. One of these strains was previously detected from three samplings of the Pa River at Xansay district, Attapeu province (Midorikawa *et al*., 2010). The DNA sequence of this ompW was homologue to *V. cholerae* O21, which we reported (GenBank: AB441168). Although this sequence differed from the common one of Nandi *et al*., (2000) it was reported also from the United States and detected even in Cambodia (Nakatsu *et al*, unpublished data). We have not detected corresponding NAG vibrios yet; however, it was thought that this DNA motif might be found widely over countries. Another was detected from the sample of the Pa Hom River in Vang Vieng, Vientiane province. This amplified DNA sequence was judged to be the usual NAG vibrios by the control and its DNA homology.

No.	Sample volume (ml)	Place/Locality of the sampling	Water	*E. histolytica*	*E. dispar*	*V. cholerae*
1	50-20000*	That Luang Swamp/Vientiane Capital	Sewage	-	(3/4)	-
2	20000*	Sewege ditch/Savannakhet	Sewage	-	-	-
3	50**	Namteun Lake/Khammouane	Lake	-	-	-
4	45	Guest house of Khong Island/Champasak	Public tap	-	-	-
5	45	Public Tube-well/Attapeu	Tube-well	(1/1)	-	-
6	50-20000*	Pa River/Attapeu	River	(1/3)	(1/3)	(1/3)
7	10-50	Mekong port of Khong Island/Champasak	River	-	(1/3)	-
8	50-20000*	Xe Kone / Attapeu	River	-	(1/3)	-
9	45	Xe Namnoi /Xekone	River	-	(1/1)	-
10	45	Xe Banhieng /Savannakhet	River	-	(1/1)	-
11	45-50	Mekhong /Khammouane	River	-	(2/2)	-
12	45	Nam Khading /Bolikhamxay	River	-	(2/2)	-
13	50**	Pa Hom River of Vangvieng/Vientiane Province	River	-	-	(1/1)
14	50**	Nam Som River of Vangvieng/Vientiane Province	River	-	-	-
15	1***	Nam Tam Chan of Vangvieng/Vientiane Province	River	-	-	-

* Concentrated sample by the method of adding DEAE (Yano et al 1993) to raw water was used for analysis.

** 10ml of the sample cultured with 1% of peptone water medium in 37C for overnight was also examined.

*** A filter paper sample containing 1 ml of the water cultured at 10 ml of 1% of peptone water in 37C overnight was used for the analysis.

Table 6. Frequency of PCR detection on en tamoeba and cholera vibrios in drinking water in Lao PRD

E. hystolytica which has pathogenicity in its genus was found only from the source of drinking water at Xansay district, Attapeu province. On the other hand, *E. dispar* was widely

distributed over the rivers in the country. In particular, in this species, frequently detected in the sewers of Vientiane, it was thought that high-level fecal contamination of this water area was demonstrated.

Bacterial detection from sampled waters: Coliform bacteria was positive in all the samples except for the sample of well water from Xansay district, Attapeu province, and the tap water of Khong Island, Champasak province. 60 bacteria stocks were recovered from the samples through this research. A tentative classification of the strains was as follows: Enterobacteriaceae including *E. coli*, *Vibrionaceae* excluding cholera vibrios, and others such as *Pseudomonas* spp. were 42, 14, and 4, respectively. The major strains of this *Vibrionaceae* were *Aeromonas hydrophila*. No cholera vibrio was confirmed in this study.

In this study it is demonstrated that NAG vibrios and two species of *Entamoeba* were genetically confirmed in water for the first time in Lao PDR.

6. Conclusion

Details of the cholera epidemic from 1993-1996 in Laos which were previously unknown have now been brought to light through records and, in particular, cases in remote mountainous areas. As a result, we now know that the cholera strain primarily responsible for the outbreak was *Vibrio cholera* O1, serotype Ogawa, biotype El Tor.

The existence of other NAG vibrios was also confirmed through research during that period, with the exception of 0139, the cause of Bengal cholera. Furthermore, it was discovered that among these vibrio bacteria, there were some strains such as O169 and O21 with a likelihood of diarrheal pathogenisis. Also, from investigations in recent years, NAG vibrios were identified over an extended period of time as contaminant strains in food available in markets in towns and cities, as well as in the water environment at a molecular level.

The NAG strains within Laos may cause new occurrences of cholera outbreaks in the future and become vehicles for drug-resistance transposons. Continued surveillance and coordinated research among neighboring countries is required to enable further surveys and studies on human and animal hosts, sewage and leftover water and food at markets.

Needless to say, water and sewer service infrastructure is of vital importance in long-term prevention of diarrhea including cholera (Watanabe *et al*., 2006). Laos has already experienced cholera epidemics. As for the prospect of prevention in the future, improvements can be seen in the habitat and infrastructure within Laos following rapid improvements in the country's economic conditions in recent years. In particular, the mountainous areas where fatality rates were high during the epidemic period from 1993-96 have been designated as focus regions in the implementation of poverty countermeasures as part of the 7th National Socio-Economic Development Plan (NSEDP) for 2011-2015. Improvements are anticipated in the installation of clean water facilities following dam construction, as well as in transportation, communication, medical care, living conditions and educational attainment. Furthermore, by training people as qualified medical technicians and introducing them to these regions, the overall standards of PHC policy implementation will be raised and consequently disease countermeasures should be considerably improved.

However, in relation to citizens' hygiene education, differences in language culture lead to problems in communicating and understanding information and knowledge on practical

disease prevention. In order to effectively promote hygiene education, we recommend the proactive introduction and utilization of IEC computer terminals making full use of information technology (IT). In addition, the introduction of a cholera vaccine for Laos will require further improvements in relevant areas, such as establishing the logistics of EPI disease monitoring specimens, creating new regional laboratories and strengthening coordination between existing regional labs and the central lab in, for example, medical technology training, together with improvements in information technology.

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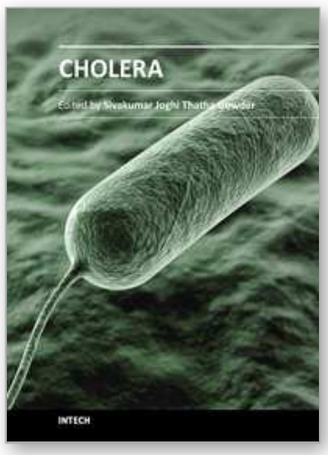
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Cholera, a problem in Third World countries, is a complicated diarrheal disease caused by the bacterium Vibrio cholerae. The latest outbreak in Haiti and surrounding areas in 2010 illustrated that cholera remains a serious threat to public health and safety. With advancements in research, cholera can be prevented and effectively treated. Irrespective of "Military" or "Monetary" power, with one's "Own Power", we can defeat this disease. The book "Cholera" is a valuable resource of power (knowledge) not only for cholera researchers but for anyone interested in promoting the health of people. Experts from different parts of the world have contributed to this important work thereby generating this power. Key features include the history of cholera, geographical distribution of the disease, mode of transmission, Vibrio cholerae activities, characterization of cholera toxin, cholera antagonists and preventive measures.

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InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

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