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Original Article

The Evaluation of Expanded Epidemic Investigations on Dengue Fever Cases in Taiwan between 2009 and 2011

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Abstract

Dengue fever, with the highest incidence rate of confirmed cases in the last ten years, is the most common acute infectious disease in Taiwan. Annually more than ten thousand people had their samples collected and tested for the dengue virus over the past three years. In order to apply dengue prevention resources more effectively, this article reviewed the expanded epidemic investigations which was conducted between 2009 and 2011. Through the data collected from notificable surveillance system and expanded epidemic investigations, the results indicated the followings: (1) an average of 3,400 suspected dengue cases were reported each year, with 1,500 confirmed cases. Most of cases were reported by medical clinics or hospitals, followed by positive cases through expanded investigations (positive contacts from dengue confirmed cases); (2) an average of 15,700 people were tested for dengue virus each year. Among them, 3,370 (21.5%) came from the surveillance system, while 12,330 (78.5%) came from expanded investigations. The former group had a positive rate of 46%, which was 23 times compared to the later group (2%); (3) less than 1% of dengue confirmed cases collected over 100 contacts through expanded investigations. Although those contacts are 16.4% of the totals, yielded a positive rate of 0.7%, much less than the overall rate of 2%; (4) through expanded investigations, contacts with symptoms were 1.8 to 41.5 times more likely to have a positive result than asymptomatic contacts. This study showed that only 2% of contacts collected from expanded investigations tested positive from 2009 to 2011, while 39.9% of the cases reported by medical clinics or hospitals tested positive. Each positive case through expanded investigations was 20 times more costly than a case

identified through reporting source of clinics or hospitals. Because of the high costs associated, expanded epidemic investigations should be conducted on people who have dengue-like symptoms, or possible dengue clusters, or who have travelled to epidemic areas, for the best results. Large-scaled mass investigations should be discouraged during outbreaks.

Keyword: dengue fever, expanded epidemic investigations, dengue confirmed case by symptom surveillance system

Introduction

Dengue fever is one of the insect-borne infectious diseases which have attracted worldwide attention. It is transmitted between people bitten by mosquitoes of the species *Aedes aegypti* and *Aedes albopictus* that carry the dengue virus. It was recorded in Chinese literature as early as 992 A.D. (Jin Dynasty), which described a dengue-like epidemic [1-2]. According to historical records of public health [3], dengue fever in Taiwan could have started in 1901, during the Japanese occupation.

Internationally, dengue epidemics were first reported in 1960, mainly in tropical and sub-tropical countries. However, with the fast development of global economy and international migration, epidemic areas quickly expanded. Nowadays dengue fever has became an endemic disease in some Western Pacific and Latin American countries [4]. Research findings and statistical data from the World Health Organization (WHO) estimate [5-8] that there may be 50 –100 million dengue infections worldwide every year. However, the latest report in Nature 2013 [9] suggested that, based on the global population in 2010, around 4 billion people each year would have been infected with dengue, although 3 billion of them might not have any obvious symptoms. These figures show that the seriousness of dengue's global expansion has obviously been underestimated. Dengue fever has been classified as a notifiable infectious disease in Taiwan since 1988. In 2002, over 5,000 indigenous dengue cases were confirmed in Kaohsiung and Pingtun areas – the worst dengue outbreak since it became a reportable disease. Between 2003 and 2012, 86 to 2,000 cases a year were logged as indigenous dengue cases – an annual average incidence rate of 4.1 per 100,000 populations.

Probably due to the effect of globalization, urbanization, and global warming, the number of reported dengue cases in Taiwan has increased gradually in recent years. In search for hidden dengue infections, local public health authorities often conduct expanded epidemic investigations around confirmed cases to collect samples from residents who might have been exposed to the virus in epidemic areas [10]. As a result, over ten thousand samples have been collected each year for testing. This study aims to investigate whether collecting samples from expanded epidemic investigations is an efficient way to utilise the limited resources for disease prevention.

Materials and Methods

1. Source of data and definition

(1) Source of data: dengue reported and confirmed cases which onset between 2009 and 2011 from notifiable diseases surveillance system, and cases with samples taken from expanded epidemic investigations.

(2) Definition:

a. A dengue reported case refers to a suspected dengue case reported by notificable disease surveillance system.

b. A dengue confirmed case refers to a reported case whose sample was tested positive for dengue virus, and matched the disease criteria, as defined by the surveillance guidelines [11].

c. A dengue confirmed case from expanded investigations refers to a case who was identified as a contact of a confirmed dengue case, and sample was collected and tested positive from the expanded epidemic investigations, and whose test result matched the criteria defined by the surveillance guidelines. Upon the confirmation of a positive test, the notificable disease surveillance system automatically produced a new reporting sheet and identified the case as a confirmed case.

d. A dengue confirmed case by symptom reporting system refers to a case which was referred by the symptom surveillance system, was tested positive for dengue virus, and matched the criteria defined by the surveillance guidelines. Upon the confirmation of a positive test result, the notificable disease surveillance system automatically produces a new reporting sheet and identified the case as a confirmed case.

e. An expanded epidemic investigation refers to the procedure when a public health authority, in order to identify more infected cases, upon receiving a report of a confirmed case, must collect samples, within 24 hours, from residents whose location was within 50 meters radius of the confirmed case, regardless of the presence of symptoms.

2. Method

After the data had been checked for errors, EXCEL formulas were used to compare and analyze test results and symptoms between groups.

Results

1. The reported and confirmed cases

Table 1 lists the statistics of indigenous and imported dengue cases between 2009 and 2011, including reported cases, confirmed cases and the positive rates.

On average, around 3,400 dengue cases were reported each year in Taiwan, 1,500 were confirmed, with a positive rate of 46%. The positive rate of indigenous cases was 44.2%, lower than the 61.3% scored by the imported cases. The major sources of reporting

(over 75%) of indigenous cases, both reported and confirmed, came from medical clinics or hospitals, followed by cases referred through expanded epidemic investigations. For imported cases, 63.2% of the cases were reported from medical clinics or hospitals and 30% were from symptom surveillance system. But for the confirmed imported cases, most (48.9%) of the cases were referred from symptom surveillance system, while 43.2% came through medical clinics or hospitals.

Table 1. Indigenous and imported dengue fever cases by source of reporting, positive rate and test results, 2009-2011

Categories of cases / Sources of reporting	Indigenous cases			Imported cases			Total		
	Reported cases*	Confirmed cases	Positive rate (%)	Reported cases*	Confirmed cases	Positive rate (%)	Reported cases*	Confirmed cases	Positive rate (%)
Hospitals/Clinics	8,245	3,275	39.7%	686	287	41.8%	8,931	3,562	39.9%
Positive cases from expanded investigation**	637	636	99.8%	44	44	100.0%	681	680	99.9%
Positive cases from symptom surveillance system	3	3	100.0%	325	325	100.0%	328	328	100.0%
Others***	135	71	52.6%	30	9	30.0%	165	80	48.5%
Total	9,020	3,985	44.2%	1,085	665	61.3%	10,105	4,650	46.0%

*Reported cases include confirmed, inconclusive, and excluded cases.
**Positive cases from expanded investigation are positive contacts from dengue fever confirmed cases by notificable disease surveillance system.
*** Include sources of reporting from foreign laborers health examination system and voluntary testing by residents.

2. Expanded epidemic investigations and confirmed cases

Table 2 shows the contacts collected from the expanded epidemic investigations between 2009 and 2011, including indigenous and imported cases.

An average of 15,700 people had been tested annually from 2009 to 2011; 78.5% of cases were collected from expanded epidemic investigations, mostly initiated from indigenous dengue cases. In these three years, the expanded investigations collected 34,931 contacts (Table 2), but only 680 cases were tested dengue-positive (Table 1). This means that for every 51 contacts collected from the expanded investigations, only one was confirmed as infected (34,931/680), with a positive rate of only 2.0% (680/34,931). Table 2 shows that on average, expanded investigations initiated by indigenous cases, regardless of whether a reported or confirmed case, collected at least 1.7 times more contacts than the investigations initiated from imported cases (3.8/2.2 or 8.2/3.5). The largest investigation from a single indigenous case collected 877 contacts, clearly a lot more than the 121 contacts collected from the largest investigation initiated from an imported case.

Ninety (90 %) of expanded investigations that started from a confirmed case had an average contacts collection of 20 or less; 43% of the confirmed cases didn't initiate any expanded investigations (40.9% of indigenous cases, and 55.8% of imported cases). However, less than 1% (33 cases) of the expanded investigations from a confirmed case collected more than 100 contacts each incidence. In total, they collected 5,740 contacts which equated to 16.4% of the total number of contacts collected. But only 40 of those contacts were tested positive, yielded a positive rate of only 0.7%. This rate was clearly lower than the average positive rate of 2%. Eighteen (18, 54.5%) of these large-scale investigations, where more than 100 contacts were collected, didn't even discover a single positive case, giving a positive rate of 0%.

Table 2. Number of cases of indigenous, imported dengue fever and expanded investigations, 2009-2011

Categories of cases / Number of people tested	Indigenous cases		Imported cases		Total	
	Reported cases*	Confirmed cases	Reported cases*	Confirmed cases	Reported cases*	Confirmed cases
Number of cases	9,020	3,985	1,085	665	10,105	4,650
Number of contacts collected from expanded investigation	34,506	32,575	2,393	2,356	36,899	34,931
Average number of contacts collected from expanded investigation**	3.8	8.2	2.2	3.5	3.7	7.5
Range of contacts collected from expanded investigation	NA	0-877	NA	0-121	NA	0-877
Number of cases which collected over 100 contacts from expanded investigations	NA	31	NA	2	NA	33

*Reported cases include confirmed, inconclusive, and excluded cases.
**the number of contacts collected from expanded investigations/the number of confirmed cases confirmed

3. Symptoms of reported cases, confirmed cases, and the contacts from expanded investigations

Table 3 displays whether those indigenous, imported cases or the contacts from expanded investigations had any symptoms when they were reported between 2009 and 2011.

For 4,457 cases, or 95.8%, of the 4,650 confirmed cases listed in Table 1 had at least one of the following symptoms: muscle pain, severe eye pain (behind eyes), rash, fever (38℃or above), headache, joint pain, low white cell count or bleeding manifestation. Of the 193 confirmed cases that didn't display any symptoms, 89.1% (172 cases) were from expanded epidemic investigations. This means a quarter (172/680) of the contacts from expanded investigations were asymptomatic infections.

Because it was not requisite to record symptoms during expanded epidemic investigations, 61.1% (22,556 cases) of the contacts from expanded investigations didn't specify whether they had any symptoms, and only 1.4% (513 cases) of them said that they had symptoms. After excluding the 22,556 cases whose symptoms were unknown, the positive rate of the contacts with symptoms was as high as 49.8%, 【(680-172)/(513+680-172)】; while the positive rate among those who didn't display any symptoms was only 1.2%, 【172/(13,830+172)】. It shows that those who had symptoms were 41.5 times more likely to be tested positive than those who didn't have any symptoms. Even after combining those cases whose symptoms were unknown with those who had symptoms (to increase the number of those with symptoms), the positive rate of those who had symptoms would still just reach 2.2%, 【(680-172)/(513+680-172+22,556)】, about 1.8 times higher than those who didn't have symptoms.

Table 3. Number of cases of indigenous, imported dengue fever and expanded investigations by symptoms, 2009-2011

Categories of cases / Presence of symptoms	Indigenous cases				Imported cases				Total			
	Reported cases*		Confirmed cases		Reported cases*		Confirmed cases		Reported cases*		Confirmed cases	
	Cases	Contacts from expanded investigations	Cases	Contacts from expanded investigations	Cases	Contacts from expanded investigations	Cases	Contacts from expanded investigations	Cases	Contacts from expanded investigations	Cases	Contacts from expanded investigations
Unknown	NA	21,929	NA	20,716	NA	627	NA	612	NA	22,556	NA	21,328
Yes	8,835	462	3,819	387	1,056	51	638	51	9,891	513	4,457	438
No	185	12,115	166	11,472	29	1,715	27	1,693	214	13,830	193	13,165
Total	9,020	34,506	3,985	32,575	1,085	2,393	665	2,356	10,105	36,899	4,650	34,931

*Reported cases include confirmed, inconclusive, and excluded cases.

Discussion

Dengue fever has an incidence rate of 4.1 per 100,000 populations in Taiwan in the last 10 years, topping the list for all acute infectious diseases. An investigation conducted in Puerto Rico [12] estimated that the annual cost of dengue fever was about 5 billion USD, the cost has not been included in the calculation of deaths. In Taiwan, central and local government agencies spend tens of millions of annual budget on dengue fever prevention programs. A research and technology development project on the economic burden of dengue fever and the application of dengue treatment and prevention programs in 2008 of Taiwan CDC [13], estimated that the total cost of Taiwan's dengue programs (including the fever screening at airports and prevention programs) averaged around one billion dollars a year. This equates to NT$6,850 per case for medical treatment and testing. Given the considerable amount of resources and staff already being contributed by cities located in high-risk areas, the social and economic burden cannot be ignored. To maximize the effectiveness of limited resources, it is even more important to evaluate the efficacy of disease prevention policies. The discussions of the findings of this study are following:

1. **The reporting of both the indigenous and imported dengue relies on vigilant doctors**
   (1) Over 90% of suspected indigenous cases were reported by doctors. Fever screenings are available at airports in order to detect suspected dengue cases, if travelers became ill after arriving later, the detection would require doctors to be vigilant by checking patients' travel history and report suspected cases actively. This demonstrates the importance of continuous training to doctors in dengue diagnosis and treatment, before the dengue season arrives, and supervision to hospitals should be enhanced. When entering the dengue season, doctors should be updated with the latest disease information through press releases or newsletters. These measures will help doctors stay vigilant, so they can report suspected cases as soon as possible. As a result, patients can be treated properly without delays, preventing unnecessary death.
   (2) The positive rate of imported cases was 1.4 times higher than the indigenous cases (Table 1; 61.3%：44.2%). This higher rate in imported cases was confounding by symptom surveillance system, because a dengue case was reported from symptom surveillance system, will soon be confirmed in notificable diseases surveillance system later. If only those cases reported from medical clinics and hospitals were considered, the positive rates between the indigenous cases and the imported cases were similar (Table 1;39.7%：41.8%).
2. **Cases reported by medical clinics and hospitals were 20 times more likely to be tested positive than cases collected from expanded epidemic investigations, showing the effectiveness when residents voluntary to visit doctors, and the benefit of early diagnosis.**

   Of the 35,000 contacts collected from the expanded investigations over last three years, only 680 cases tested positive. On average, one of every 51 contacts collected would be tested positive – a positive rate of merely 2.0%. In comparison, the positive rate of those cases reported by medical clinics and hospitals was 39.9% (Table 1), which was 20 times (39.9/2.0) more effective. Moreover, of the 680 cases that tested positive from expanded investigations, 75% of them had dengue-like symptoms. Had these 508 symptomatic cases been more vigilant and seen a doctor early, the costs of having to screen 27,000 people in order to find 508 cases would have been saved. This result affirms the importance of educating residents about dengue-like symptoms, as it not only prevents or stops the spread of dengue outbreaks, but also avoids unnecessary waste of precious prevention resources and delays in receiving treatment.
3. **Contacts collected from expanded investigations with symptoms were more likely to be tested positive than contacts without symptoms**

   Most of the dengue confirmed cases already displayed symptoms when they were tested. Only 4% of those infected didn't have any symptoms. This is because more than 75% of dengue cases were reported by medical clinics and hospitals, and only those who already had symptoms would visit a doctor. Analysis of the contacts collected from

expanded investigations has found that the positive rate of the contacts collected from those who already had symptoms ranged from 2.2 to 49.8%, but the rate of the contacts collected from those who didn't have any symptoms was only 1.2% or less. This shows that those cases that had symptoms were 1.8 to 41.5 times more likely to be infected than those without symptoms. When resources are limited, it will be the most effective if only collected samples from those who display symptoms.

4. Quarantine plays an important mechanism in preventing viruses from other countries

Half of the confirmed imported cases were detected by quarantine measures at international ports. This shows that fever screening at international ports has became an important mechanism in preventing imported infectious diseases, not just for dengue fever. In order to improve the efficacy of testing, Taiwan CDC started using the dengue NSI express testing kit at airport screening centers from June 2008. This has greatly helped with early diagnosis and treatment of patients, and preventing dengue from entering to communities. As our citizens frequently travel to Southeast Asian countries for business and leisure, any infection brought back by travelers would affect our dengue control. It is recommended that citizens be alert when they travel abroad (especially those high risk groups such as new immigrants and foreign laborers), ensuring that they follow personal hygiene measures. This will not only lower the risk of imported infections spreading into local communities, but will also reduce the costs involved when imported cases cause local outbreaks.

5. Large-scaled mass investigations to collect samples is not cost-effective

(1) The main differences between the number of contacts collected from investigations initiated from indigenous cases and the imported cases are:

a. On average, investigations initiated from indigenous cases collected over 1.7 times more contacts than from imported cases, as shown in Table 2, (3.8/2.2 or 8.2/3.5).

b. The rate of imported confirmed cases didn’t initiate any expanded investigations was 1.4 times than from indigenous confirmed cases(Table 2;55.8/40.9=1.4）.

c. The rate of indigenous confirmed cases with more than 100 contacts collected resulted in 2.6 times than imported cases(Table 2; 31/3,985：2/665）.

d. The largest investigation initiated from a single indigenous case collected seven times more contacts than a single imported case (Table 2 ; 877/121).

These findings show that as soon as an indigenous case occurred, local health authorities needed to actively search for the source of infection in order to stop the virus from spreading further. As a result, these expanded investigations were more costly than those on imported cases. The reasons for such a difference in the amount of contacts collected from expanded investigations between indigenous and imported cases could be that imported cases had passed the viremic stage and was less likely to cause local infections when they entered the country. As a result, no further investigations were required when imported cases were diagnosed. Another reason

might be that when the patients were infected while travelling, the possible exposed people were limited to other members and the tour guide in the same tour, so there was no need to collect too many contacts.

(2) 33 confirmed cases collected more than 100 contacts from ecpanded investigations. Of the total 5,740 contacts collected, only 40 were found to be dengue positive, a rate of merely 0.7% which was clearly lower than the rate of the overall expanded investigations (2.0%). More than half (18 cases) of confirmed cases collected more than 100 contacts did not detect any positive dengue case from 3,262 contacts collected, included the largest investigations in 2010 (877 contacts) and 2011 (283 contacts). Although the average number of contacts collected from the expanded investigations initiated from indigenous cases was 1.7 times higher than from imported cases (Table 2, 3.8/22), the positive rates between the two groups were similar. This proves that large-scaled expanded investigations would not find more positive cases. Local governments may have special reasons to expand the investigations, such as the need to respond to the first dengue case, or because of the threat of potential dengue clusters. However, this study has found that large-scale mass sampling not only won't help in finding the source of infection, but will also waste valuable resources. It is recommended that contacts should be collected from targeted groups such as suspected clusters, people who have been travelling to epidemic areas, or those who have displayed dengue-like symptoms. Large-scale sampling should be avoided whenever possible, especially in areas where resources are limited. During epidemic outbreaks, careful consideration should be given before expanding the targets and areas of epidemic investigations.

In addition to the reasons listed above, it is also often difficult to ensure that residents who are located within a 50 meter radius of a confirmed case, always be collected their samples. As a result, Taiwan CDC amended in its "Dengue Fever Prevention Guidelines" and re-defined the scope of expanded epidemic investigation to include "monitoring the health of residents who live within a 50-meter radius of the active area of the confirmed cases. Blood samples should be collected from residents who have displayed symptoms in order to identify the source of infection" in 2013. Because the positive rate of reporting resources from medical clinics and hospitals is 20 times higher than the expanded epidemic investigation, it is also recommended that local health authorities continue educating the public about dengue symptoms, so that residents can watch out for possible symptoms and visit doctors early for proper treatment. These measures will help to preserve precious healthcare resources.

Limitations

Data used in this study came from the Taiwan CDC's notifiable diseases surveillance system, which is a passive reporting system. The numbers of reported and confirmed cases did not represent the actual prevalence of infections and were clearly under-estimated.

Symptoms recorded from expanded epidemic investigations were reported by patients themselves and over 60% of those investigated did not answer questions regarding their symptoms. As a result, the value of its efficacy could only be expressed as a range. These two issues were the main limitations of this study.

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A Family Outbreak of Cholera in Changhua County

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Abstract

In September 2012, two indigenous cholera cases occurred in Changhua County. An elderly couple in coastal areas was hospitalized due to vomiting, diarrhea and watery stools. *Vibrio cholerae* was isolated from the stool specimens. Toxigenic strains, biotype E1 Tor and serogroup O1 serotype Ogawa, were confirmed by Taiwan CDC. The investigation revealed that the clam farming couple had a simple diet, mostly home-made ingredients, rarely dined out. The same strain was isolated from family flush toilet. Non-O1 and non-O139 strains were isolated from residual clams and farm site. Non-toxigenic O1 Ogawa was isolated from two aquatic species. PFGE typing showed strains from two cases and the toilet have the same fingerprinting pattern, but obviously differ from non-toxigenic strains. The infection might due to the couple eat raw, not fully cooked or contaminated clams, along with high susceptibility. High diversity of *V. cholerae* was discovered in the aquaculture farming place, indicating *V. cholerae* (toxigenic strains) should have widely lived in waters and the surface of aquatic species in estuaries. The prevention control of cholera in Taiwan should emphasize on health education, clam and fish should be fully cooked, avoid eating raw food. Prevent cross-contamination of cooked and uncooked food. In addition, the leftover food should be refrigerated in case of *V. cholerae* contamination.

Keywords: Indigenous cholera, *V. cholerae* O1 Ogawa, PFGE

Introduction

Cholera has caused seven worldwide pandemics since 1817 and killed millions. In early times, the epidemic occurred in Asia and Africa. In Taiwan, 4 epidemics have occurred since 1912. The latest case took place in 1962. Later, only sporadic cases happened in the following 50 years. The incubation period of cholera ranges between 12 hours and 5 days. The typical symptom is “rice water” like diarrhea. Sometimes in its severe forms, dehydration, acidosis and circulatory system failure occur. The major infection source comes from the intake of

polluted food and water. According to statistics, the infectious dose of *V. cholerae* O1 has been estimated to be 10^5-10^8 cells, but could be as low as 10^3 in the presence of achlorhydria [1]. Therefore, most infected cases are asymptomatic or only mild diarrhea. Patients with the use of gastric acid inhibitor and gastrectomy, the elderly, the people with chronic diseases and cancer, and those who are immuno-compromised are highly susceptible to cholera infection.

V. cholerae has been divided into over 200 serogroups on the basis of its lipopolysaccharide (LPS) somatic antigen. In history, only O1 and O139 serogroups cause pandemics, whereas those non-O1 and non-O139 serogroups cause diarrhea. Some strains produce cholera toxin, but never caused epidemics. *V. cholerae* O1 can be divided into 3 serotypes called Ogawa, Inaba, and Hikojima. *V. cholerae* O1 can be classified into two biotypes, classical and E1 Tor, by biochemical tests. In 1992, *V. cholerae* O139 was first recognized in South Asia as a cause of cholera epidemic. Molecular epidemiological studies suggest that the O139 strains have evolved from O1 E1 Tor strains. This is done through lateral transfer genomic island [2]. After *V. cholerae* enter the body, most of it well be killed in gastric acid. The survival cells colonize in small intestine and release cholera toxin. The genes for the toxin are encoded within the genome of a filamentous bacteriophage, CTXφ [1]. Therefore, cholera toxin can be transmitted through lateral transfer between strains.

Materials and Methosd

A. Case profile

In September 2012, a central medical center reported two suspected cholera cases who lived in coastal areas. The husband (Case 1) suffered diarrhea, vomiting, loss of appetite at end of August. On September 6, he was sent to the nearby hospital because of a fever, continuous watery stools, general weakness, and fall. Later, the husband was transferred to a medical center. On September 8, he was very sick and stayed in ICU with hemodialysis. He has diabetes, hypertension, and chronic renal insufficiency. In 2011, he also suffered Stevens Johnson syndromes. His wife (Case 2) had mild dementia. On September 6, she was hospitalized with similar symptoms. *V. cholera* was isolated from stool specimens of these two cases and was reported on September 11 and 12. Strains were sent for testing to Taiwan CDC on September 13. Later these were confirmed as toxigenic *V. cholerae* Ogawa O1. Since they had no domestic or foreign travel history and no eating out records, so to trace the source of infection, the survey was focused on home and aquaculture environment. Moreover, the couple engaged in clams farming, and the food source mainly came from poultry and aquatic products, either from self or relatives.

B. Epidemiological Investigation and Laboratory Testing

1. Environmental investigation and specimen collection

On September 13, the Third Branch (Taiwan CDC) along with Changhua County Public Health Bureau and centers went to the couple's house and clam farming site for field

surveys and sampling of contact, food, and environment. On September 17, the second environmental sampling was proceeded. All specimens include 2 cases, 2 contacts, 11 environmental specimens and 7 farming specimens. Tests were done at Center for Research and Diagnostics (Taiwan CDC). In addition, 7 food specimens (oyster, rice cakes, and rice dumplings) and clams were sent to Central Center for Regional Administration, Taiwan Food and Drug Administration (TFDA). Another non-toxigenic strain of *V. cholerae* O1 Ogawa (isolated from a diarrhea case who lived in another township of Changhua County in September) was included in this study (Table).

Table. Results of the tested specimens

Specimen types	Sample from	Test results	Pathogenic characteristics
Human	Case 1 stool (Isolate)	*V. cholerae*	O1, Ogawa/El Tor/ctx (+)
	Case 2 stool (Isolate)	*V. cholerae*	O1, Ogawa/El Tor/ctx (+)
	Granddaughter (Contact)	Not detected	
	Son (Contact)	Not detected	
	Another case (Isolate)	*V. cholerae*	O1/Ogawa/ctx (-)
Home environment	Flush toilet	*V. cholerae*	O1, Ogawa/El Tor/ctx (+)
	Groundwater well	Not detected	
	Kitchen sink swab	Not detected	
	Cutting board 1 (for meat)	Not detected	
	Cutting board 2 (for vegetable)	Not detected	
	Kitchen knife (large)	Not detected	
	Kitchen knife (medium)	Not detected	
	Kitchen knife (small)	Not detected	
	Coconut water & clam soup	Not detected	
	Kitchen faucet (tap water)	Not detected	
	Water filter faucet in kitchen	Not detected	
Farming environment /crab & shell	Farm water-Son	Not detected	
	Farm water -Case 1	Not detected	
	Clam	*V. cholerae*	Non-O1, non-O139
	Clam-farming water	*V. cholerae*	Non-O1, non-O139
	Snails	*V. cholerae*	Non-O1, non-O139
	Donax variabilis	*V. cholerae*	O1, Ogawa/ctx (-)
	Crab	*V. cholerae*	O1,Ogawa/ctx (-)
Food*	Clam 1	*V. cholerae*	Non-O1, non-O139
	Clam 2	*V. cholerae*	Non-O1, non-O139
	Clam 3	*V. cholerae*	Non-O1, Non-O139
	Clam 4	*V. cholerae*	Non-O1, Non-O139
	Clam 5	*V. cholerae*	Non-O1, Non-O139
	Oyster	Not detected	
	Rice cakes & rice dumplings	Not detected	

* Tested by TFDA

2. Identification and typing of cholera strain:

(1) Specimen culture:

a. Human rectal swab was directly cultured in thiosulfate citrate bile salt sucrose (TCBS) medium.

b. Environmental wipe swab was first cultured in 5 ml of alkaline peptone water (APW) with 1% NaCl (pH 8.6) (Creative Media Products, Ltd., Taiwan) at 37℃, shaking at 150 rpm for 12-16 hours. After enrichment, use a cotton swab dampened with the test liquid and streak onto TCBS medium.

c. Water samples: 100ml of 5X concentrated APW with 1% NaCl (pH 9.2) was added to 400 ml of water samples. After shaking culture at 37℃, 150 rpm for 12-16 hours, use a cotton swab dampened with the test liquid and streak to TCBS medium. Thereafter, take 40 ml of the first enrichment broth coupled with 10 ml of 5X concentrated APW with 1% NaCl (pH 9.2) for second enrichment. After shaking culture at 37℃, 150 rpm for 12-16 hours, use a cotton swab dampened with the test liquid and streak to TCBS medium.

(2) Strain identification:

a. Observe the morphology of colonies on TCBS medium, all suspected yellowish colonies were picked and sub-cultured on tryptic soy agar (TSA); also inoculated to the triple sugar iron agar (TSIA), lysine iron agar (LIA) and sulfide indole motility agar (SIM) for biochemical tests. Read the results after incubated at 37℃ for 16-18 hours.

b. Use API20E biochemical identification kit (BioMerieux, France) for biochemical tests.

c. Serotyping：Take *V. cholerae* O1 and O139 antisera to operate the slide agglutination test. If O1 antiserum has positive agglutination, use Ogawa and Inaba antisera for further agglutination test to confirm the serotype.

d. If biochemical and serological test confirm the presence of *V. cholerae* O1 or O139, then toxin gene identification would be tested.

(3) Cholera toxin gene identification: Take a single colony on TSA to prepare the suspension, add two sets of specific primers [3] to do polymerase chain reaction (PCR). The primer sets used can amplify the specific *V. cholera*e *hly*A gene, as well as cholera toxin gene *ctx*AB. *V. cholerae* 569B was used as a positive control strain.

(4) Pulsed-field gel electrophoresis (PFGE): The standardized PulseNet PFGE protocol for *V. cholerae* was followed [4]. Chromosomal DNA was digested separately by restriction enzymes *Not*I and *Sfi*I. Next, DNA band was separated by PFGE. The resulting gel was stained with ethidium bromide (EtBr) before being photographed and the image stored as a TIFF file.

(5) Pattern analysis: Software BioNumerics 6.6 (Applied Maths, Kortrijk, Belgium) was used to process data standardization, alignment, comparison, and phylogenetic tree construction.

Results

1. Demographic information of cases:

(1) In the family, the couple lives with a son and two grandchildren. No foreign laborer. Recently, no foreign trave l and history of exposure to foreign laborers are found. However, the exposure to the household poultry is evident. The couple lives mainly on household poultry, vegetables, fish, and clams. They rarely ate out or bought food from

the neighborhood. Since other family members do not eat at home, the diet before the couple felt sick cannot be clarified. Besides, Case 2 cannot tell the food freshness because of mild dementia. Even if there are spoiled foods in the house, those were cooked. As for Case 1, he does not like fish, but drinks clam soup. In addition, Case 1 ate sweet rice dumplings and rice cake plates before illness. According to him, he ate fried clams from his son's farming, and felt the strange smell. Some of these clams were given to relatives, but a few of them did not eat owing to something wrong.

(2) There are two kinds of water usage in the house. First, the tap water with a simple filter in the kitchen. Second, there is a groundwater well in front of the house, water is pumped manually. Therefore, the well water is not exposed to the surface. When Case 2 prepares meals, she used to wash foods with groundwater in front of the house and cook with tap water. There were two kinds of clams and one cooked tilapia stored in the refrigerator.

(3) Case 1 and his son deal in clam-farming. There are three clam-farming sites. One is rented out. Two are used by themselves. The son's clam-farming site is 500-1000 meters away from the house. Case 1's site is 1-2 km away from the house. Two months ago, some clams from Case 1 site were harvested, and we were not sure if these were kept until the couple felt sick. Besides, some of the clams from the son's site were harvested on Sep. 3. At that time, the couple felt sick. Of course, the clams from other family members and relatives are regularly given to the couple.

2. Laboratory testing and genomic typing

As shown on Table 1, three toxigenic *V. cholerae* O1 serotype Ogawa strains were isolated from two cases and the flush toilet specimen. Non-O1 and non-O139 strains were isolated from clam farm water, aquatic species, and leftover clams.

Obviously, the *V. cholerae* contained in specimens is quite complex since the colony morphology on media is polymorphic. Also, two non-toxigenic strains of *V. cholerae* O1 Ogawa were isolated separately from one crab and one *Donax variabilis* from the farming site. Another non-toxigenic strain of *V. cholerae* O1 Ogawa isolated from Changhua township in September was included in the analysis. The three toxigenic strains showed 100% similarity in the PFGE pattern (Figure) indicated these come from the same origin. The non-toxigenic strain from another case and three toxigenic strains are close but with difference. The patterns of the two non-toxigenic strains isolated from aquatic species and toxigenic strains are apparently different.

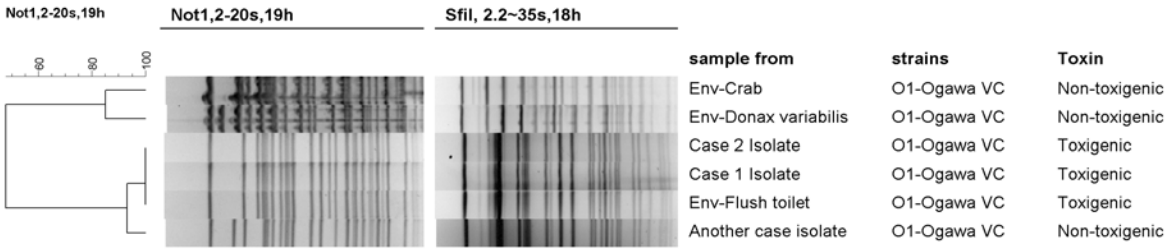

Figure. The phylogenetic tree of *V. cholerae* O1 Ogawa strains

3. Prevention and control measures

The Disease Control Section at Changhua County Public Health Bureau started monitoring the severe diarrhea case at clinics and medical institutions in the four neighborhood townships after two cases were notified; also interviewed any resident for suspected symptoms, and enhanced sanitation and sterilization. No severe diarrhea case or cluster was found recently. Later, a cholera case in another town was reported. The isolated strain was non-toxigenic *V. cholerae* O1 serotype Ogawa. Moreover, the Food Safety Section instituted the movement control for the clam-farming site; the Hygiene Inspection Section investigated the downstream vendors for product flow directions. After confirming no further toxigenic strain was found, the ban then lifted.

Discussion and Suggestion

World Health Organization (WHO) reported that the global cholera incidence gradually increases from 2005. The latest epidemic occurred in Haiti after the earthquake in 2010. In areas with insufficient sanitation and sewage treatment, if *V. cholerae* or patient's excretions and vomitus contaminate the water, it is very easy to cause a large scale outbreak. Haiti has no cholera for more than 50 years before, however, after the earthquake, the insufficient sanitation facilities and infrastructures were destroyed. The sudden outbreak of cholera was out of control because of no clean drinking water available. To investigate the cause of cholera, the team from the United Nations (UN) studied the strains phylogenetic relatedness through whole genome sequencing and discovered that the outbreak strain had the closest phylogenetic relationship to a Bangladesh strain [5]. It was speculated that UN Peacekeepers might be the source of outbreak. Presumably introduced by the affected peacekeeping soldiers from cholera endemic areas in Asia. The contaminated water ran into a neighboring river from their base. Afterwards, the disease was spread out rapidly. This proved the ultimate importance of water control in prevention of cholera.

In developed countries with sufficient sanitary facilities, cholera happens through the contaminated food, which mainly comes from seafood. Based on the investigation, the infection source for Case 1, being a susceptible population, might be related to the diet of incompletely cooked clams or the food contaminated by *V. cholerae*. Case 2 might be infected by the same food source, or by human-to-human transmission. Investigation on the previous sporadic cases showed the difficulty in tracking down the history of food consumption, plus the food specimens were not available, making it not easy to find out the infection source. From the investigation data, we can find that in addition to eating incompletely cooked seafood, cross-contamination of raw and cooked food in containers is a pathway to be easily ignored. To future health education, fully cook seafood, separating eating utensils for raw and cooked food are main points for the public.

In this study, many different types of colony morphology of *V. cholera* were isolated from the water specimens and aquatic species in clam-farming sites. These colonies mainly belong to non-toxigenic non-O1, non-O139 strains. It was thus proved that *V. cholerae* originally existed in estuary water and aquatic organisms. In this investigation, more than 10 different colonies from every specimen were picked up for identification, and two non-toxigenic strains of *V. cholerae* O1 Ogawa was isolated from one *Donax variabilis* and one crab specimen. It was speculated that toxigenic O1 and O139 strains should have existed in waters, but not a large amount. Presently with advanced bacterial isolation and culture techniques, specific O1 and O139 strains can been isolated from non-O1, non-O139 strains, or by using molecular biology techniques, toxigenic O1 and O139 strains can be sensitively detected; these approaches are powerful tools for ecological research of *V. cholerae*. To search for the presence of toxigenic *V. cholerae* in our estuary water environment, the studies conducted by academic and fishery research institutions from 4 different counties/cities found their presence at aquaculture farming sites but were non-toxigenic [6]. However, researchers mentioned that in addition to the sensitivity of testing techniques and validity of sampling, *V. cholerae* would change the composition of the bacterial cells under unfavorable environments, and further enter into the viable but non-culturable stage, thus no colony formation on the media can be seen, thus creating pitfalls in epidemic prevention testing [6]. On the other hand, Taiwan and other neighboring countries have frequent trade exchanges, and the underground economy is booming. Another investigation should be focused on the use or smuggling of aquatic products, whether these cause the illness of Taiwanese and the risk of contamination of waters.

During 1997-2000, outbreak of cholera O139 cases occurred in Taiwan due to eating raw soft-shelled turtle eggs and was clearly evidenced. However, the sporadic cases after 2000 were all unable to be identified. These cases occasionally took place in northern, central and southern Taiwan. Once a cholera case was identified, the disease prevention units would actively track down the origin and process the testing, but eventually in vain. We would image that because people usually dine out, instead of home cooking, the leftover foods may be littered right away and leave no residual food for further investigation. However, at a current trading speed and distance of importing and exporting food, any single infection source may show up in different time and space and affect people. On the contrary, even if no positive case around the infected people, it cannot be concluded that the outbreak comes to an end. Facing the changeable daily consumption patterns and food traceability system, we need to build up a mechanism of cooperation and investigation among government units in order to effectively search for the origin of a disease and to stop the food-borne disease from spreading. In addition, the techniques of quarantine and disease prevention must be updated. Nowadays, the emphasis on measures of investigating imported products in many countries has placed the food safety as the No.1 policy. Therefore, the exporting countries have reformed their production policy and testing techniques in order to gain the sale markets.

The PFGE was used to analyze the phylogenetic relationship between the case and environment strains in this study. The purpose is to observe if the surroundings of the clam-farming site might be the infectious source. Later, if typing of the recent cholera case strains is necessary, it is feasible in techniques to set up a pattern database. Since Taiwan is not a cholera endemic country, there are rare cases in recent years, plus the infectious source is uncertain, and similar deficiency in strain patterns from other countries also evident. We cannot offer much substantial help to prevention units beside the typing results since we have limited data and statistics for analysis.

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