

The Taiwan Epidemiology Bulletin series of publications is published by Centers for Disease Control, Department of Health, Taiwan(R.O.C.) since Dec 15, 1984.

Publisher : Feng-Yee Chang

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Suggested Citation :

[Author].[Article title].Taiwan Epidemiol Bull
2010;26:[inclusive page numbers]

education directed at the susceptible population and the education of physicians on diagnosis and notification of suspected cases. 2. Investigators should do their best to collect detail information on food origin, food preparation, eating utensil, food storage, and eating habits of the reported cases, and health status of close contacts and neighborhood people. 3. National laboratory should establish data bank for native strains to facilitate the comparative analysis with strains from other countries. 4. Agriculture authority periodically conduct survey on environment and fishery of farm ponds; food sanitation authority routinely monitor on safety of marketed marine product; and authorities in charge of agriculture, food sanitation, and disease control should establish a channel for mutual communication and work together to assure citizen's health and safety. 5. Department of disease control will have to collect and obtain information on environmental surveillance and risk assessment for coastal areas along Taiwan, Penghu, Kinmen, and Matzu.

Keywords: cholera, infection source, investigation

Introduction

Cholera, a kind of acute bacterial enteritis, is characterized clinically by sudden onset, profuse painless watery diarrhea in its severe form, and nausea and profuse vomiting in early stage. Most infected cases are asymptomatic or experience only mild diarrhea. However, cases even in asymptomatic carrier can transmit the disease to others.

No epidemic cholera has occurred in Taiwan since an epidemic caused by *Vibrio cholerae* serogroup O1 in 1962. Since then only sporadic cases were reported except few cluster outbreaks. A total of 18 indigenous cholera cases were identified during the period of 1997-2009. In order to understand the possible infection source and relevant risk factors, this study has reviewed the reports of epidemiologic investigations for the 18 cases and has collected information on environmental surveillance, field survey, and reports from domestic and foreign countries. The purposes of this study are to explore the epidemiologic characteristics, risk factors, potential environment sources, and improvable points in epidemiology investigation concerning the 18 indigenous cases, to provide a reference in investigation of infection sources and prevention control for the future.

Materials and Methods

1.Study sample

Eighteen cholera cases reported to the Communicable Disease Surveillance System of Taiwan CDC during the period from 1997 to 2009 were included in the study. All cases

were noted no experience of travel abroad before illness and were laboratory confirmed the bacteria strain as toxigenic *Vibrio cholerae* either serogroup O1 or O139.

2. Study method

We first collected data recorded in the Communicable Disease Surveillance System (including demographic information, clinical symptoms, disease history, and medical records) and information on epidemiological investigation (including exposure of risk factor, environmental, diet, and community investigation) of these cases, and reviewed relevant literatures. The data were analyzed by Microsoft Excel software and performed the descriptive statistical analysis. Moreover, a comparison between the information obtained in this study and relevant field investigations and researches in foreign countries was conducted.

Results

Chronological description of indigenous cases (Table 1)

In 1997, a male case, aged 71, with gastrectomy history, lived in Kaohsiung City was identified. This case became ill two days after he returned from “a love trip to Meinung soft-shelled turtle farm site” arranged by a company, where he ate raw soft-shelled turtle eggs. The investigation showed that a total of 287 persons participated in the trip and 245 specimens obtained from the participants were all tested negative for *V. cholerae* by National Institute of Preventive Medicine (incorporated into Taiwan Centers for Disease Control in 1999) except the specimen from the case. Infection source investigation revealed that the soft-shelled turtle farm site had been

contaminated with *Vibrio cholerae* serogroup O139. In the same year, the same serotype of *V. cholerae* was also isolated from farm sites in Jiaushi Township of Ilan County and Yanpu Township of Pingtung County and, in the next year, from that in Shinpi Township of Pingtung County. The source investigation for the contaminated farm sites co-conducted by agriculture and health authorities indicated that the water used for farm ponds was negative for *V. cholerae*, but the same serotype strain of *V. cholerae* as that from cases was recovered from the trash fish used for feeding turtle.

In 1999, a total of 3 cholera cases were identified, all aged more than 70 years. Two of them lived alone in Hsinchu County and Taichung City, respectively. Investigation showed that both of them were living in a condition with inadequate sanitation status and poor personal hygiene. Moreover, one had gastrectomy, the other suffered from chronic disease and long-term use of gastric acid inhibitor. The strains of *V. cholerae* O1 serotype Ogawa and O139 had been recovered from the two cases, respectively. Specimens from residual food and water kept in refrigerators used by the patients were all negative for *V. cholerae*, while specimen from flush toilet of the patient living in Taichung City was positive for *V. cholerae*. The infection sources for both of the patients were unknown. The specimen from the third patient, aged 79-year who lived in Kaohsiung City, was confirmed to be positive for *V. cholerae* serogroup O139. The patient ever consumed raw and alcohol-soaked soft-shelled turtle eggs before onset of the disease. Therefore, the soft-shelled turtle eggs were proved to be the source of infection.

Table 1. Summary of indigenous cholera cases occurred in Taiwan during 1997~2009

No.	Year of onset	Sex	Age of onset	Sero-groups	Occupation of patient	Underlying disease or risk factors	Infection source	Places of infection	Date of onset	Date of diagnosis	Main symptoms
1	1997	M	71	O139	Unemployed	Gastrectomy	Ingestion of raw soft-shelled turtle eggs	Kaohsiung County	8/26/1997	8/27/1997	Diarrhea
2	1999	F	80	O1 Ogawa	Unemployed	Gastrectomy	Unknown	Hsinchu County	6/8/1999	6/11/1999	Vomiting, diarrhea
3	1999	M	73	O139	Unemployed	Diabetes, hypertension, anti-acid drug user	Unknown	Taichung City	6/28/1999	7/7/1999	Vomiting, diarrhea
4	1999	M	79	O139	Unemployed	Unknown	Ingestion of raw soft-shelled turtle eggs and Chinese herbal medicine soaked alcohol	Kaohsiung City	11/20/1999	11/24/1999	Unknown
5	2000	M	22	O139	Student	None reported	Ingestion of raw soft-shelled turtle eggs	Pingtung County	6/11/2000	6/13/2000	Vomiting, diarrhea, dehydration
6	2000	M	21	O139	Student	None reported	Ingestion of raw soft-shelled turtle eggs	Pingtung County	Unavailable	6/17/2000	No symptoms
7	2000	F	23	O139	Unknown	None reported	Caregiver of cholera case	Pingtung County	Unavailable	6/22/2000	No symptoms
8	2000	M	80	O139	Unemployed	Gastrectomy, duodenal ulcer	Ingestion of food contaminated by raw soft-shelled turtle during preparation	Miaoli County	6/20/2000	6/20/2000	Nausea, vomiting, diarrhea, dehydration, acute renal failure, acidosis
9	2000	M	28	O139	Unavailable	None reported	Ingestion of food contaminated by raw soft-shelled turtle during preparation	Miaoli County	Unavailable	7/5/2000	No symptoms
10	2000	F	20	O139	Unavailable	None reported	Ingestion of food contaminated by raw soft-shelled turtle during preparation	Miaoli County	Unavailable	6/29/2000	No symptoms
11	2000	F	52	O139	Unavailable	None reported	Ingestion of food contaminated by raw soft-shelled turtle during preparation	Miaoli County	Unavailable	6/29/2000	No symptoms
12	2005	F	72	O1 Ogawa	Housekeeper	Hypertension	Unknown	Tainan County	6/16/2005	6/20/2005	Nausea, vomiting, diarrhea, acute renal failure, acidosis
13	2005	F	77	O1 Ogawa	Housekeeper	Lung cancer (chemotherapy)	Unknown	Tainan City	6/24/2005	7/4/2005	Diarrhea
14	2006	F	58	O139	Farmer	None reported	Unknown	Tainan County	5/10/2006	5/17/2006	Diarrhea, vomiting, acute renal failure, acidosis
15	2008	F	67	O1 Ogawa	Housekeeper	Chronic renal disease (dialysis), hepatitis C	Unknown	Kaohsiung County	5/25/2008	5/30/2008	Diarrhea
16	2009	F	72	O1 Ogawa	Housekeeper	Heart disease, diabetes, hypertension	Unknown	Taipei County	9/5/2009	9/11/2009	Diarrhea, vomiting
17	2009	M	32	O1 Ogawa	Unemployed	Liver cirrhosis	Unknown	Yulin County	9/8/2009	9/12/2009	Diarrhea, abdominal pain, nausea, vomiting
18	2009	F	81	O1 Inaba	Unemployed	hypertension	Unknown	Tainan County	9/19/2009	9/24/2009	Diarrhea

Two cluster infections, including a total of seven cases that were all positive for *V. cholerae* O139, occurred in 2000. The index case of the first cluster event was a male college student aged 22 years. He traveled together with his girlfriend to visit a classmate living in Yanpu Township of Pingtung County where the classmate's family was running a soft-shelled turtle farm. Four people, including index case, his girlfriend, classmate, and classmate's father, had consumed mixture of raw soft-shelled turtle eggs (around 30 eggs) with chicken essence. After wards, the index case took 40 soft-shelled turtle eggs with him when he left for home, he ate some raw on the arrival day and three days later after arrival. In fact, the index case had suffered from diarrhea 3~ 4 times on the third day after returning home but he still ate the rest of eggs without cooking on the next day, and additional diarrhea 4~ 5 times occurred. Negative result was obtained from specimens of his girlfriend and classmate's father, while specimens from his classmate and older sister of the index case were positive although they were all asymptomatic. Since his sister did not have the same exposure history as the index case in food consumption, she might have gotten infection from the index case while she took care of him during his sickness. Environmental investigation revealed that the soft-shelled turtle farm pond had been contaminated with *V. cholerae* O139. Another cluster event involved a wedding banquet held in Gungguan Township of Miaoli County. The index case is an elderly male with gastrectomy history, aged 80 years. He became sick two days after he attended the wedding banquet with his families, where the soft-shelled turtle had been served.

Three additional positive cases (all asymptomatic) were identified from members had meal at the same table with the index case. Specimens from the chef and waiters working for the banquet were all negative. Environmental specimens from soft-shelled turtle farm pond where supplied the soft-shelled turtle for the banquet were also negative. It is speculated that the cluster infection might have resulted from the cross contamination of other dishes served for the banquet by the raw soft-shelled turtle during food preparation.

There were two cholera cases in 2005. They are both female older than 70 years of age. The first case, a patient with hypertension and living in Tainan County, was considered to get infection from three-color steamed egg that she bought from food stand (another seafood stand selling fresh oyster, clam, and fish just located at the opposite side of it) in a market near her home and consumed without reheating two days before onset. The patient recalled that the three-color steamed egg had stinky odd smell and looked slimy outside of it when she ate, but she still ate all of it. Specimens obtained from products sold by the food stands, including salted fish, raw oyster, clam, and three-color steamed egg, were all negative. However, specimens taken from the drain outlet of kitchen sink in house of the case were positive. It is speculated that the sink should have been contaminated when the patient was washing those food. Therefore, the infection source for this case might be related to food [3]. The second case is a lung cancer patient receiving chemotherapy and living in Tainan City. Although the strain of *V. cholerae* O1 serotype Ogawa was isolated from both

first and second case, the epidemiologic investigation showed that no association existed between the two cases. Foods for the second case were bought at nearby traditional market and usually prepared by her daughter-in-law. She ever ate sashimi roll and fish before onset of the disease but no residual foods were available for laboratory test. The infection source was unknown.

A female case aged 58 years living in Tainan County involving agriculture-related job was confirmed to be positive for *V. cholerae* O139 in 2006. Although she did not have a history of preexisting disease, she had a poor personal hygiene, such as often neglecting to wash hands after work, frequently eating food placed under room temperature for a long time without reheating. She ever ate stewed chicken claws with spoiled sour taste two days before getting sick, but no residual for laboratory test. Specimens collected from foodstuff (including sarsaparilla soft drink, milk, bubble milk tea, and tapioca flour) in the patient's refrigerator were all negative for *V. cholerae*. The patient had attended banquet in her village 5 days before onset of disease, where cold dish, mixed fruit dish, shrimp salad, and ice cream were served, but no residual food items were available for test since it happened two weeks earlier. Moreover, other participants of the banquet were unable to be identified. However, no other suspects were detected through the interview of residents in the village and survey of nearby clinics. The infection source was unknown.

In 2008, a case of a 67-year-old female with a history of chronic renal disease (on kidney dialysis) and hepatitis living in

Kaohsiung County was identified to be infected with *V. cholerae* O1 serotype Ogawa. Investigation revealed that the patient usually did not maintain a good personal hygiene practice. She ever consumed chháu-á-ké (not reheated before eating) and boiled sea snail at a street stand in Dashe Township of Kaohsiung County three days before onset of disease. Specimens obtained from residual sea snail and families were all negative. The infection source was unknown.

There were three cases in 2009, which no epidemiological association existed among them and infection sources were all unknown. The first case is a 72-year-old female who lives in Taipei County. She had a history of heart disease, hypertension, and diabetes, and did not have a good personal hygiene practice. She ever ate stale food made from bitter melon with fermented black bean (no residual for test) 2~3 days before onset of disease. Specimens collected from water of saltwater aquarium tank in the patient's home and from stool of patient's son living with her were all negative. The second case is a 32 years old male with history of liver cirrhosis living in Yunlin County. The causative agent isolated from both first and second cases were *V. cholerae* O1 serotype Ogawa. Epidemiological investigation showed that the patient usually consumed cooked food and drank boiled water or packed soft drink. No foods or drinks were identified to be related to the case. Specimens taken from patient's families living with her were all negative. The third case is an 81-year-old female living in Tainan County with history of hypertension and did not have a good personal hygiene practice, with a dirty and messy house. She ever ate leftover

milkfish cooked two days ago before onset of disease but no residuals were available for test. Specimens collected from foods stored in patient's refrigerator, surface of wall and shelf inside the refrigerator, and environment were all negative except those from flush toilet were positive. Specimens from patient's families living in the same house were also negative. The pathogenic agent isolated from this case was *V. cholerae* O1 serotype Inaba.

Statistical analysis of indigenous cholera cases

Of the 18 indigenous cholera cases identified during 1997 to 2009, eight of them are male and 10 are female. The group of 61 years of age and older accounted for the highest percentage of cases, 55.6% (10/18 cases), followed by the group of 0~30 years of age, 27.8% (5/18 cases), and then 31-60 age group, 16.6% (3/18). The analysis of regional distribution showed that the Kaohsiung-Pingtung region experienced the highest percentage of cases, 33.3% (6/18), followed by the southern region and northern region, both 27.8% (5/18), and then middle region and Taipei region, both 5.6% (1/18). The symptom of diarrhea represented the highest percentage of cases, 66.7% (12/18), followed by symptom of vomiting, 44.4% (8/18), and then asymptomatic infection, 27.8% (5/18). The average length of interval between date of the onset of symptoms and date of diagnosis was 4.6 days, with a shortest interval of 0 day and longest interval of 10 days. The analysis of underlying disease showed that patients without underlying disease accounted for 38.9% (7/18) of the cases, 38.9% (7/18) with underlying chronic

disease, and 16.7% (3/18) of cases with history of gastrectomy. For behavioral risk factors, patients with poor personal hygiene (without washing hands before meals and after toilet use) and inadequate food sanitation practices (without reheating cooked food or leftover foods before eating) represented 33.3% (6/18) of cases. Patients infected with strain of *V. cholerae* serogroup O139 and serogroup O1 recorded 61.1% (11/18) and 38.9% (7/18) of the cases, respectively. Investigation showed that 22.2% (4/18) of the cases presumably infected through the participation of parties, 22.2% (4/18) through banquets, and 55.6% (10/18) in the household. The modes of transmission for these cases include food consumption in 8 cases (serogroup O139), contact in 1 case (serogroup O139), and unknown in 9 cases (7 cases with serogroup O1, 2 cases with serogroup O139). No cholera infections were found to be related to the contamination of drinking water source.

Discussions

Cholera cases identified during the last twelve years were mainly indigenous cases. Based on the epidemiological characteristics, in the first phase, from 1997 through 2000, eleven cases were identified, which were mainly caused by the strain of *V. cholerae* serogroup O139 (10 cases), mostly became ill after the participation of parties or banquets, even resulted in cluster infections in some cases, and were almost transmitted through the consumptions of raw soft-shelled turtle eggs or other food contaminated by raw soft-shelled turtle during preparation. The source investigation co-conducted by agriculture and health authorities for the contaminated

soft-shelled turtle eggs indicated that the water used for farm ponds was not contaminated but the same serotype of *V. cholerae* from cases was recovered from the trash fish used for feeding turtle in farm ponds, such as those in Jiaushi Township of Ilan County, Meinung Township of Kaohsiung County, and Yanpu Township of Pingtung County. The trash fish was suspected to be smuggled from cholera endemic areas. Of the eleven cases, about half of them have come up with cholera-related symptoms but another half appeared no symptoms. Most of the cases with symptoms were those with a history of gastrectomy or chronic disease and with an older age, while asymptomatic cases were all without underlying disease and with a younger age. In the second phase, from 2005 through 2009, seven cases were identified, mainly caused by the strain of *V. cholerae* O1 serotype Ogawa (6 cases), and were all epidemiologically unrelated cases, and their infection sources were all unknown. Among the seven cases, except that one patient with history of cirrhosis was 32 years of age, other cases were all elderly people aged more than 58 years (average age 71.1 years) and most of them have underlying disease, including hypertension, diabetes, chronic renal disease, or cancer.

Previous studies used to consider that human was the main reservoir of the disease and transmission occurred mainly through ingestion of water or food contaminated by patient's excreta. However, studies in recent years indicated that *Vibrio cholerae* can survive naturally in water, especially in brackish water or in estuary water, and can live in symbiotic relationship with plankton [2]. It

also can survive in harsh environments by entering the viable but non-proliferative state through the hibernation process, and become active again and continually grow and proliferate under environments with adequate temperature, salinity, and PH value [4]. Global climate change has influenced the growth of plankton and has certainly affected the growth of *V. cholerae* that has a symbiotic relationship with plankton. Experts believed that the cholera cases continually occurred in India subcontinent and other areas should be highly related to the environmental factor [2]. Moreover, studies conducted in Australia, Bangladesh, and USA also found that *V. cholerae* can live in natural environments [5]. When the pathogenic *V. cholerae* can continually exist in the natural environments, it can be transported to areas located thousand miles apart through the discharge of ship's ballast water [4]. Epidemiologist has observed a cholera case occurred in Texas, USA in 1973, from a fisherman who was very rarely infected with *V. cholerae* O1 serotype Inaba. Five years later, a cholera outbreak caused by the serotype of *V. cholerae* identical to the strain of previous case occurred in the same place because of consuming non-fully cooked seafood, leading to infection of 24 cases. It is recognized that the infection source of the outbreak originated from US Gulf of Mexico. Later investigation also found that the strain of *V. cholerae* O1 serotype Inaba has been native to US Gulf of Mexico [4].

On environmental surveillance, investigation proved that *V. cholerae* serogroup O1 or O139 also can be found in other sites besides soft-shelled turtle farm ponds identified in earlier time. During the

period of 1994 through 1998, a total of eight non-toxicogenic strains of *V. cholerae* serogroup O1 have been isolated from specimens taken from water in Taichung Harbor and Kaohsiung Harbor by National Quarantine Service (The antecedents of the Taiwan Centers for Disease Control). In a study granted by the Council of Agriculture, the researchers conducted an investigation to fish/shrimp farm ponds in 2005. Of the 91 farm ponds surveyed, *V. cholerae* serogroup O1 was isolated from 11 water specimens (from ponds for tilapia, eel, grouper, white shrimp, and other seawater fish) and 5 fish/shrimp (including tilapia, grouper, and white shrimp) specimens, which cholera toxin gene (ctx gene) was detected in strain of *V. cholerae* serogroups O1 in 4 of 11 water specimens (from ponds for grouper, white shrimp, and other seawater fish) and 1 of 5 fish/shrimp specimen. *V. cholerae* serogroup O139 was isolated from 5 water specimens (from ponds for tilapia, goldlined seabream, and other seawater fish) and 7 fish/shrimp (including grouper and cobia) specimens [6]. In 2005, Chen et al. detected non-toxicogenic *V. cholerae* serogroup O1 from farm ponds located in Yulin County and Kaohsiung County and non-toxicogenic *V. cholerae* serogroup O139 from farm ponds in Yulin County and Pingtung County [7]. Although these strains are all non-toxicogenic, previous studies indicated that ctx gene can be transferred from toxicogenic strain to non-toxicogenic strain through bacteriophage, converting non-toxicogenic strain into toxicogenic strain [8]. Taiwan CDC conducted survey on *V. cholerae* for 17 fishing harbors distributed along the coast of Taiwan Island in 2009. Although no strains of *V. cholerae* have been

detected in this survey, global warming, climate change, and international transport could change the environment from long term perspectives. Therefore, the results of environmental surveillance will always be an important indicator for providing alert on environmental contamination.

As part of analysis on relationship between cholera occurrence and environmental factors, Chen et al. conducted molecular typing analysis for strain of *V. cholerae* O1 serotype Ogawa isolated from two cases occurred in Tainan County in 2005, from cases imported from Indonesia in 1990 and 1995, and from China in 1999. They found that the genetic similarity reaches as high as 95% between cases in Tainan County and those imported from Indonesia in 1990, and 85% identical between cases in Tainan County and those imported in 1995 and 1999. Chen et al. speculated that the strain of *V. cholerae* imported from Indonesia in 1990 should have entered environment and survived in a dormant form. Then the dormant form may infect human opportunistically through the consumption of contaminated seafood or drinking water, leading to the infection of two cases in Tainan County [9]. The toxicogenic strain of *V. cholerae* O1 serotype Inaba isolated from case occurred in Tainan County in 2009 was the first indigenous case since 1965. Other strain of *V. cholerae* O1 serotype Inaba isolated from cases identified in Kaohsiung County in 2003 and in Tainan County in 2006 are all non-toxicogenic, except an imported case from Thailand in 2003. Therefore, the infection source for case in 2009 was probably originated from either the revival of dormant strain or the genetic change of non-toxicogenic

strain in the environment.

Based on currently available data, we postulated the hypothesis that *V. cholerae* serogroups O1 and O139 may have natively existed in our natural environments. To verify this hypothesis, we need to continually conduct environmental surveillance (especially in costal areas and fish farm ponds). In addition, molecular epidemiologic analysis should be performed to prove whether the strains of *V. cholerae* O1 serotype Ogawa isolated from imported cases (11 cases) occurred during 1990~1995, and in 1999, 2002 and 2004 were associated with the indigenous cases infected by the same serogroup in 1999 and 2005, and during 2008~2009. However, except environmental factors, another important infection source that can not be neglected for this country is the commercial trade on the sea between the fishermen from this country and those from China and illegally smuggled fishery products from China since cholera is currently occurring in provinces along the coast of China, such as provinces of Zhejiang, Fujian, Guangdong, and Hainan island [10]. Therefore, once fishery products smuggled from China and without undergoing quarantine inspection get into this country, we can not exclude the possibility that they may become an important factor of causing sporadic cholera cases. This scenario of cholera occurrence is our second possible hypothesis. If authorities in charge of food sanitation could routinely implement inspection of fishery product sold in the markets, the alert for the possible occurrence of cholera outbreak could be noticed earlier and the evidence supporting infection source of cholera cases could be provided. Another

hypothesis for the occurrence of indigenous cholera cases is that the infection source may originate from imported cases infected when they were traveling to cholera epidemic areas for sightseeing, visiting relatives, or family reunion of foreign worker in Taiwan. When these cases were asymptomatic or mild cases, they might have introduced cholera agent and infected others but not be detected. The verification of this hypothesis requires early diagnosis and early notification of cholera cases by physician as well as epidemiological investigation for cholera infection.

On vehicle of cholera transmission, the infections in developed countries usually occurred due to the ingestion of contaminated food. The food contamination may occur through the attachment of *V. cholerae* on body surface of fish or shellfish in the water environment or through cross-contamination from other contaminated food because of poor food sanitation practices during the preparation process. When the contaminated food was not stored properly (not refrigerated), even a little amount of bacteria can proliferate at an exponential rate under adequate environmental conditions and reach to the amount of being able to cause disease within several hours [4]. In an epidemic situation, the investigation in identifying the source of cholera infections is usually conducted by applying a case-control study to find out the implicated risk factors (or vehicles), but, in situation of sporadic infections, this method is generally unsuitable and to find out infection source is more difficult. World Health Organization (WHO) has analyzed the possible vehicles linked to *V. cholerae* infections and indicated that the transmission

usually took place through the contaminated drinking water, ice products made from contaminated water, contaminated food containers, shellfish harvested from contaminated water, vegetables or fruits irrigated or washed with contaminated water, or food cross-contamination during preparation or after cooking. For example, *V. cholerae* in contaminated foods, including milk, rice, lentils, potatoes, beans, eggs and chicken meat, will proliferate within several hours under room temperature [11]. In addition, according to the reports published in the most recent decade all over the world, the infection sources attributed to cholera epidemic in cholera-infected areas (such as Africa, South-east Asia, and China) include the contamination of lake water, river water, reek water, pond water, well water, reservoir water, or drinking water, as well as the contamination of lettuce, soft-shell turtle, raw shrimp, shrimp sauce, chicken claws, marine algae, fish, marine crustaceans, and mollusks in the farming sites or processing plants, during the preparation because of cross-contamination or improper storage, or through fly vector.

Data from USA showed that no local epidemics have occurred for the past 100 years in the USA except some small scale outbreaks and most of the cases are sporadic. According to the US CDC statistics, a total of 29 indigenous cholera cases infected with *V. cholerae* serogroup O1 have been identified during the period of 1996 through 2005. Investigation revealed that 20 (69%) of the 29 cases were associated with consumption of seafood (including crab, shrimp, and oyster) and the rest (31%) of the 29 cases whose sources of infection were unknown.

Hurricanes Katrina (in August) and Rita (in September), the super strong hurricanes in nearly 100 years, continually attacked USA in 2005, leading to severe flooding in Bay areas of southeast USA. Three weeks after the devastation of Rita hurricane, a couple in Louisiana State were infected with *V. cholerae* O1 serotype Inaba. The main symptoms for husband who had history of hypertension and diabetes included severe watery diarrhea and dehydration, while his wife presented only a mild diarrhea. Investigation showed that the cause of infection was consumption of uncooked or contaminated shrimps. They bought the shrimps from local fisherman and cooked for about five minutes and subsequently placed some of the cooked shrimps into a container used for holding raw shrimps to wait for them to cool down and then ate them. Two additional persons eating the shrimps together with the couple also displayed mild symptoms but did not seek medical treatments. The cholera outbreak eventually implicated improper food preparation and food treatment, or poor living conditions and inadequate sanitation after hurricane devastation [12]. Therefore, US experts advised that shellfish should be well cooked or boiled continuously for more than 10 minutes and then should be placed in a clean container to avoid recontamination [13-14].

There have been no cholera epidemics in Taiwan since 1962, when an epidemic caused by *V. cholerae* serogroup O1 occurred. During this period, most of the infections were sporadic cases except few clusters. The cases identified in recent five years were among elderly age, had chronic underlying disease, and did not

have good personal hygiene or food sanitation. Specifically, cases occurred in 2005 (2 cases) and 2009 (3 cases) were relatively more in number than those in other years. Moreover, both of the cluster infections happened in 2005 and 2009 occurred within 4~42 days after 612 flooding and 88 flooding in southern Taiwan, respectively. Although these cases were not coming from those who suffered from flooding, the occurrence of these infections was very likely to be associated with food contamination by environmental strains combined with poor personal hygiene and inadequate food sanitation. Investigation conducted by other countries revealed that toxigenic strain of *V. cholerae* can survive in natural environment and infect persons through ingestion of contaminated food, even a small amount of bacteria, under adequate environment, can proliferate and reach to the amount sufficiently threatening human health and causing disease among immuno-compromised susceptible hosts. The reasons why infection sources for cholera cases could not be identified through epidemiological investigation are partly because the recognition of infection sources for sporadic case has always been difficult and the insufficient information on food consumption provided by patients. And it would be helpful for investigation of infection sources if physicians could keep vigilance and sensitivity over diagnosis of suspect cholera cases and improve notification efficiency; the average interval between date of onset and date of being diagnosed is 4.6 days and the longest interval is 10 days. In reviewing past reports of epidemiological investigation concerning cholera cases, we found that the investigation usually focused more on the issue of whether

patients have consumed raw seafood (fish, clam, etc.), vegetables, fruit juice, or ice products during the incubation period of the disease but less on the issues of whether cross-contamination have occurred during the preparation of food consumed by the patient or whether the patient has a good food sanitation practices, such as using clean eating utensil, eating heated food, and storing food well under refrigerated conditions. Therefore, if epidemiological investigation could pay more attention on the data collection and analysis concerning these issues, it would be useful for the identification of infection sources and the establishment of disease control and prevention strategy. In addition, trace (place of product origin, and the health situation of food seller and whether he/she has history of traveling abroad and employs foreign workers) of suspicious food material and food items (no matter whether it is marine products or not), investigation of close contact (including history of traveling abroad and occurrence of suspicious symptoms), survey of missing cases living in neighborhood areas with detected cases (cases with suspicious symptoms living near reported cases), and sampling of specimens from the suspicious leftover or food material consumed by the reported cases would also be useful in the recognition of infection sources.

Recommendations

1. To protect residents from infection, people vulnerable to cholera infection (such as patients with history of gastrectomy, receiving long-term anti-acid therapy, immuno-compromised patients, with chronic disease, and in elderly population)

should be considered as target population for providing health education and be taught especially about the importance of maintaining a good personal hygiene and food sanitation practices, and fully cooking and heating food before eating, to avoid ingesting food with potential risk of cross-contamination.

2. Health authorities should be sensitive to the occurrence of cholera infection and be capable of timely detection of index cases. Local governments where cases occurred should strengthen the education of physicians on diagnosis and notification of suspected cases.
3. In order to clarify infection source or infection route, investigators should do their best to collect detail information on food origin, food preparation, eating utensil, food storage, and eating habits of the reported cases, close contacts and neighborhood people, and to take specimens from suspicious leftover or food material associated with cases.
4. In order to investigate the origin of cholera strain responsible for the infection, national laboratory should establish data bank for native strains to facilitate the analysis of DNA fingerprinting and drug resistance of the strains and the comparison with strains existed in other countries. Also, these can be a reference for forming disease control policy.
5. To eliminate the risk of contaminated marine products to the health of citizens, we suggest that agriculture authority periodically conduct survey on pathogenic agents in environment of farm ponds and fishery raised in ponds, and food sanitation

authority routinely implement monitoring on safety of marketed marine product. Therefore, authorities in charge of agriculture, food sanitation, and disease control should establish a channel for mutual communication and work together to assure citizen's health and safety.

6. Global warming, climate change, and international transport will continually affect the occurrence and distribution of pathogenic agent in natural environment of this country. Therefore, if more cholera cases were occurring, department of disease control would have to immediately collect and obtain information on environmental surveillance and risk assessment for coastal areas along the islands of Taiwan, Penghu, Kinmen, and Matzu.

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- ## ***Ehrlichia chaffeensis* Infection in Rodent Ticks—Kinmen, 2009**

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- Abstract**
- Samples used in this study were collected between September 28 -30, 2009 on Kinmen island. 110 ticks at larva or nymphs stages, including *Rhipicephalus haemaphysaloides*, *Haemaphysalis* sp., and *I. granulatus* were gathered from 26 small rodents including moles. The ticks were separated into 29 pools according to their species and hosts. The DNA from ticks was extracted, amplified by nested PCR, and compared with 16s ribosomal RNA genes. The results showed that 2 pools of the samples (KM29-35T and KM29-40T) had 99% and 100% homology to *Ehrlichia chaffeensis*. The two pools were from nymphs of *R. haemaphysaloides* and *I. granulatus* found on two *Rattus losea exiguus* captured around Sango Folk Village. The minimum infection rate (MIR) of *E. chaffeensis* in Kinmen was 1.8% (2/110) and in Sango Folk

Village was 11.1% (2/18).

Keywords: *Ehrlichia chaffeensis*, nested PCR, rodent parasitic ticks, Kinmen

Introduction

Human monocytic ehrlichiosis (HME) is caused by *E. chaffeensis*, an absolute intracellular parasitic bacterium of Rickettsiales, family Anaplasmataceae, transmitted by ticks [1]. The bacterium was first identified in Arkansas, USA, in 1986, from a blood smear of a then unknown disease caused by ticks. Aggregates were found in monocytes in the blood smear. The disease was initially diagnosed as Rocky Mountain Spotted Fever, but aggregates of *E. chaffeensis* was later identified. *E. chaffeensis* was originally thought to be an animal pathogen [2], which was isolated from cell culture and named in 1991. *E. chaffeensis* is seen as an emerging disease. Symptoms of human patients range from asymptomatic seroconversion to death [3]. Incubation period is about 1-2 weeks with an average of 9 days. Symptoms of infection include fever (>95%), headache (60-75%), myalgia (40-60%), nausea (40-50%), arthralgia (30-35%), anorexia (30-80%) [4,5], GI upset, lymphadenopathy, splenomegaly, and skin rash. Some cases might have severe symptoms including renal failure, CNS symptoms and respiratory failure. While biting, ticks will penetrate mouthparts into the skin. The mouthparts should be clamped as soon as possible to remove the ticks. Do not squeeze the tick in order to prevent fragments of their mouthparts from leaving in the skin [6]. Vector of *E. chaffeensis* in the USA was *Amblyomma americanum* found on canines, deer and sheep

[7, 8]. However, ticks infected are different in other geographic areas, such as *Haemaphysalis yeni* found in China [9] and *Ixodes ricinus* found in Russia [10]. Besides America, Europe, Thailand, and Northeast Asia [11, 12, 13], *E. chaffeensis* is also found in spleens of rodents in Fujian [9]. Cases have been reported from Inner Mongolia, China [14, 15]. In recent years, traffic between China and Taiwan has increased significantly. Kinmen is close to China, and sharing common rodent species with Fujian [15,16]. Although there are vector ticks survey reports concerning spotted fever and Lyme disease in Kinmen [17, 18], no study about HME has been published in Taiwan. *E. chaffeensis* can be transmitted through multiple tick species. Since the wilderness in Kinmen is large, wild rodents in Kinmen have a vector tick prevalence rate of 54.9% [18]. Hence, in this study ticks were gathered from rodents and identified by nested PCR in order to understand infection rates of rodent ticks in Kinmen as a reference for clinical follow-up.

Materials and Methods

1. Collection locations

Rodents were captured in a 3-day period between September 28 - 30, 2009, from following 4 areas, wild fields, pasturages, sea ports, and military camps. The wild fields included area near Sango Folk Village, Yang-Di, and Chung-Lin. Pasturages included Mr. Car pasturage, Lin-Do pasturage, Bishan range pasturage, Sha-Mei Red Flag pasturage, and Kinmen Livestock Research Institute. Sea ports included Liaoluo Wan Port and Fishing Village. Military camps included camps at Tai-Wu Mountain, Shiau-Chin, Chung-Lin and Yang-Di area.

2. Tick sampling

Rodents were anesthetized via ip by 0.05-1.0 ml Zoletil 50 (Virbac Lab. France, 10-fold dilution) and checked by brushing. Once ticks were found, ticks and the attached skin were removed by small scissors, kept in boxes with 40 ml of gypsum and charcoal (9:1), and labeled with host species. Ticks were kept in 4°C during transportation.

3. Testing of *E. chaffeensis*

(1) DNA extraction

Ticks were sorted according to their species and hosts, put into 1.5ml autoclaved centrifuge tubes, sterilized with iodine, washed three times with sterile PBS (pH 7.0), and homogenized by Roche MagnaLyser (Roche Diagnostics GmbH, Germany) with nuclease-free beads, added 300 µl PBS, shake at 6,500 rpm for 1 min., samples were then centrifuged at 1,200 rpm for 15 min. 200 µl supernatant was used to extract DNA by using the QIAamp DNA Mini Kit (QIAGEN GmbH, Hilden, Germany).

(2) Primers

Published sequences (ECC, ECB) were used to selectively amplify *Ehrlichia* spp. 16S rRNA genes [19]. HE1, HE3 [9],

ECH16S-17F, and ECH16S-97R [20] were used to amplify *E. chaffeensis* genes. Primers were synthesized by Genomics Corp. ECH16S-38PRO [20] is a real-time PCR probe synthesized by ABI Corp. (Table 1). Positive control was *Ehrlichia chaffeensis* strain Arkansas 16S ribosomal RNA gene (479bp) with primers ECC and ECB from Genomics Corp.

(3) Nested PCR amplification

16S rRNA was first tested by 47.5 µl of mixture of 36.25 µl sterile Q water, TaKaRa PCR buffer 5.0 µl (TaKaRa-Bio, Japan), TaKaRa dNTP Mixture 4.0 µl, 10µM primer (ECC) 1.0 µl, 10 µM primer (ECB) 1.0 µl, and TaKaRa Taq™ HS 0.25 µl, plus 2.5 µl of DNA. PCR reaction was done with the following condition: 94°C 5 min, and 40 cycles of 94°C 1 min, 60°C 1 min, and 72°C 1 min, followed by 72°C 10 min and ended at 4°C. Real time PCR quantification was done by 19 µl of primer and enzyme mixture (9 µl sterile Q water, TaqMan®Universal PCR Master Mix 10 µl (Roche, USA), 10 µM primer (ECH16S-17F) 0.4 µl, 10 µM primer (ECH16S-97R) 0.4 µl, probe ECH16S-38PRO 0.2 µl) and 1 µl of products

Table 1. Primers used to detect *Ehrlichia* spp. in ticks in Kinmen area

Primer	Sequence (5'-3')	Target	Gene	Size(bp)	Reference
ECC	AGAACGAACGCTGGCGGCAAGCC	<i>Ehrlichia</i> spp.	16S	478	19
ECB	CGTATTACCGCGGCTGCTGGC				
HE1	CAATTGCTTATAACCTTTTGGTTATAAAT	<i>E. chaffeensis</i>	16S	389	9
HE-3	TATAGGTACCGTCATTAICTTCCCTAT				
ECH16S-17F	GCGGCAAGCCTAACACATG	<i>E. chaffeensis</i>	16S	81	20
ECH16S-97R	CCCGTCTGCCACTAACAATTATT				
ECH16S-38PRO	FAM- AGTCGAACGG ACAAT TGCTTA TAACCTTTTGGT-TAMARA				

from the PCR mentioned above. The reaction was done with an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, Calif. USA) with the following condition: 95°C 10 min and 40 cycles of 95°C, 15 sec and 57°C, 1 min. The results were shown as averages of Ct values from triplicates. To further confirm the sequence of amplicons, 1 µl of the PCR products was added to 49 µl of mixtures of sterile Q water 37.75 µl, TaKaRa PCR buffer 5.0 µl, TaKaRa dNTP Mixture 4.0 µl, 10 µM primer (HE1) 1.0 µl, 10 µM primer (HE3) 1.0 µl and TaKaRa Taq™ HS 0.25 µl and amplified with the following condition: 94°C 5min, 40 cycles of 94°C 1 min, 52°C 1 min, 72°C 1 min, followed by 72°C 10 min and ended at 4°C. Amplicons of the secondary PCR were analyzed by electrophoresis in the TAE buffer. Products of 389bp were extracted by the QIAquick gel extraction kit (QIAGEN GmbH, Hilden, Germany), confirmed again by electrophoresis, and sequenced by the

Genomics Corp.

Results

From the 10 locations, including Lin-Do pasturage, Mr. Car pasturage, Kinmen Livestock Research Institute, Bishan range pasturage, wild fields near Sango Folk Village, Sha-Mei Red Flag pasturage, camps at Tai-Wu Mountain, Shiau-Chin, Chung-Lin, and Yang-Di area, 110 ticks at larva or nymphs stages, including *Rhipicephalus haemaphysaloides* (n=69), *Haemaphysalis* sp. (n=3), and *I. granulatus* (n=38) were gathered from 26 small rodents including *Suncus murinus*. Ticks found on *S. murinus* were all *I. granulatus*. Those found on *Rattus norvegicus* were all *R. haemaphysaloides*. Three species of ticks were found on KM29-11 *Rattus losea exiguus* (Table 2). 29 samples were amplified by primers ECC and ECB. After real time PCR selection, 2 pools had positive reactions. The average Ct of triplicates were 15.2 and 28.4, respectively (Fig. 1).

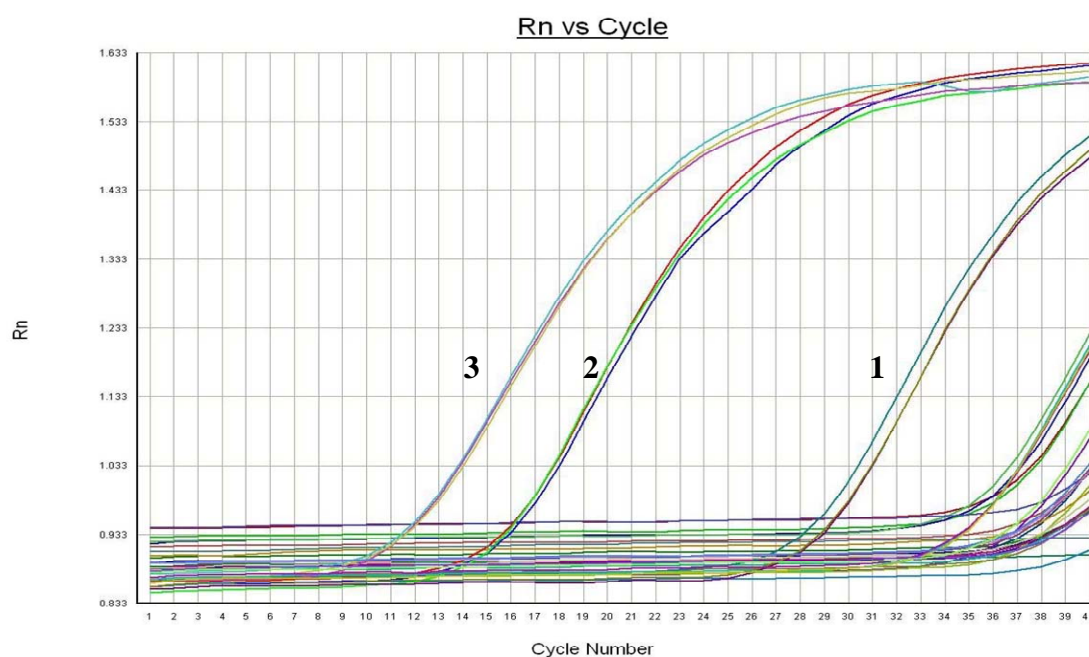


Figure 1. Results of real-time PCR reaction of *E. chaffeensis* in ticks in Kinmen area
1.KM29-40T, 2.KM29-35T, 3.16S rRNA

Table 2. Samples of ticks from rodents in Kinmen area

Samples	Ticks	Species	Numbers	Place found	Hosts
1	KM29-10T1	<i>I. granulatus</i>	1 nymph	Lin-Do pasturage	<i>Rattus losea exiguus</i>
2	KM29-10T2	<i>R. haemaphysaloides</i>	18 larvae, 1 nymph	Lin-Do pasturage	<i>Rattus losea exiguus</i>
3	KM29-11-T1	<i>Haemaphysalis sp.</i>	2 nymphs	Lin-Do pasturage	<i>Rattus losea exiguus</i>
4	KM29-11-T2	<i>I. granulatus</i>	2 nymphs	Lin-Do pasturage	<i>Rattus losea exiguus</i>
5	KM29-11-T3	<i>R. haemaphysaloides</i>	2 nymphs	Lin-Do pasturage	<i>Rattus losea exiguus</i>
6	KM29-13T	<i>R. haemaphysaloides</i>	4 larvae, 2 nymphs	Lin-Do pasturage	<i>Rattus losea exiguus</i>
7	KM29-14T1	<i>R. haemaphysaloides</i>	3 larvae	Lin-Do pasturage	<i>Rattus losea exiguus</i>
8	KM29-14T2	<i>I. granulatus</i>	1 nymph	Lin-Do pasturage	<i>Rattus losea exiguus</i>
9	KM29-15	<i>R. haemaphysaloides</i>	4 nymphs	Mr. Car pasturage	<i>Rattus norvegicus</i>
10	KM29-16T	<i>R. haemaphysaloides</i>	2 nymphs	Mr. Car pasturage	<i>Rattus losea exiguus</i>
11	KM29-17T	<i>R. haemaphysaloides</i>	3 nymphs	Mr. Car pasturage	<i>Rattus losea exiguus</i>
12	KM29-18T	<i>R. haemaphysaloides</i>	5 larvae, 6 nymphs	Mr. Car pasturage	<i>Rattus losea exiguus</i>
13	KM29-27T	<i>R. haemaphysaloides</i>	2 larvae	Kinmen Livestock Research Institute	<i>Rattus norvegicus</i>
14	KM29-31T	<i>R. haemaphysaloides</i>	2 larvae, 3 nymphs	Bishan range pasturage	<i>Rattus losea exiguus</i>
15	KM29-33T	<i>I. granulatus</i>	4 larvae	Sango Folk Village	<i>Suncus murinus</i>
16	KM29-35T	<i>R. haemaphysaloides</i>	2 nymphs	Sango Folk Village	<i>Rattus losea exiguus</i>
17	KM29-36T	<i>R. haemaphysaloides</i>	1 nymph	Sango Folk Village	<i>Rattus losea exiguus</i>
18	KM29-39T	<i>R. haemaphysaloides</i>	2 nymphs	Sango Folk Village	<i>Rattus losea exiguus</i>
19	KM29-40T	<i>I. granulatus</i>	2 nymphs	Sango Folk Village	<i>Rattus losea exiguus</i>
20	KM29-41T	<i>R. haemaphysaloides</i>	5 larvae	Sango Folk Village	<i>Rattus losea exiguus</i>
21	KM29-42T1	<i>R. haemaphysaloides</i>	1 larva	Sango Folk Village	<i>Rattus losea exiguus</i>
22	KM29-42T2	<i>Haemaphysalis sp.</i>	1 larva	Sango Folk Village	<i>Rattus losea exiguus</i>
23	KM29-50T	<i>R. haemaphysaloides</i>	1 nymph	Sha-Mei Red Flag pasturage	<i>Rattus norvegicus</i>
24	KM29-67T	<i>I. granulatus</i>	1 nymph	Shiau-Chin	<i>Suncus murinus</i>
25	KM29-77T	<i>I. granulatus</i>	3 larvae	Tai-Wu Mountain	<i>Suncus murinus</i>
26	KM29-88T	<i>I. granulatus</i>	6 larvae	Chung-Lin	<i>Suncus murinus</i>
27	KM29-92T	<i>I. granulatus</i>	6 larvae	Yang-Di	<i>Suncus murinus</i>
28	KM29-98T	<i>I. granulatus</i>	4 larvae	Yang-Di	<i>Suncus murinus</i>
29	KM29-99T	<i>I. granulatus</i>	8 nymphs	Yang-Di	<i>Suncus murinus</i>

For further confirmation, PCR amplicons of primers ECC/ECB were amplified by primers HE1 and HE3. Sequencing results of the 389bp, when searched through the NCBI website (<http://www.ncbi.nlm.nih.gov>), shows that the 2 samples had genomes of *Ehrlichia chaffeensis* str. Arkansas, which has a homology of 100% and 99% with strains from Korea, Japan, China and the USA with differences of only 1-2 bps (Table 3).

The two pools were KM29-35T (nymphs of *R. haemaphysaloides*) and KM 29-40T (nymphs of *I. granulatus*) from ticks on *Rattus losea exiguous* captured in wildfield around Sango Fork Village. The results showed that the minimal infection rate of *E. chaffeensis* in ticks in Kinmen was 1.8% (2/110) and the minimal infection rate of *E. chaffeensis* in ticks in Sango Fork Village was 11.1% (2/18).

Discussion

According to the literature, HME is caused by *E. chaffeensis* distributed in America, Europe, and Thailand; and

transmitted by ticks among animals. Human infection is usually sporadic, and there is no human-to-human transmission [1, 13]. In Taiwan, other than canine monocytic ehrlichiosis [21], no human cases have been reported. *E. chaffeensis* has been found in spleens of rodents in Fujian [9], which is close to Kinmen. Although transmissibility of vector ticks has not been established, Kinmen has a large area of wild fields and a higher density of wild rodents (mainly *Rattus losea exiguous*). Our earlier studies of rodent ticks in 2007 and 2009 found the average tick index of each rodent was 2.7 and 1.9, respectively. The major ticks found on rodents in Kinmen are *R. haemaphysaloides* and *I. granulatus* (unpublished data). A study, the molecular identification of vector ticks in Taiwan, done in 2003 by Taiwan CDC, revealed that the major two ticks in Kinmen were *I. granulatus* and *Rhipicephalus sanguineus*. The later was found on dogs [18]. Whether *E. chaffeensis* found on vector ticks, although the transmissibility has not been established, could

Table 3. Differences of 16S rRNA genes among *Ehrlichia chaffeensis* in ticks in Kinmen area

Strain	Nucleotide Position			*Homology
	45	96	302	
<i>E.chaffeensis</i> Arkansas	A	C	A	-
KM29-35T	A	C	A	100%
E.c.(China_GQ499945)	A	C	A	100%
KM 29-40T	G	C	A	99%
E.e.(USA_EEU96436)	A	C	G	99%
E.sp.(China_DQ324547)	A	C	G	99%
E.sp.(Japan_AB196303)	A	T	G	99%
E.sp.(Korea_GU075697)	A	C	G	99%

*Compared with 16S rRNA gene of *E.chaffeensis* Arkansas

become an emerging disease requires attention in public health studies. Two positive pools (KM29-35T and KM29-40T) found in wild field near Sango Folk Village were all from *R. losea exiguous*. Splens of their hosts (KM29-35 and KM29-40), when checked with nested real-time PCR, had Ct values of 13.7 and 38.7, respectively, showing that the hosts were also infected. Although the bacterium is known to be transmitted by ticks, infection and vertical transmission between ticks is still not clear [22]. This study data have shown that ticks and *R. losea exiguous* in Kinmen have *E. chaffeensis* infection as in Fujian [9]. Infection of *R. losea exiguous* and other rodents by *E. chaffeensis* remain to be investigated.

Acknowledgements

The authors thank the Department of Defense for funding support and the help from rodent investigation team by Institute of Preventive Medicine, National Defense Medical Center, Taiwan.

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