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An acute gastroenteritis outbreak of Vibrio parahaemolyticus O4:K55 in Nursing College, Thailand

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Abstract. A cluster of acute gastroenteritis among nursing students was noticed on 13th September 2005. Between 13th and 17th September 2005, a retrospective cohort study was then conducted to identify the most likely cause of gastroenteritis at a nursing college in Bangkok, Thailand. Self-administered questionnaires, interviews, environmental investigations, and rectal swabs from all participants were carried out. In the investigation, 98.9% female nursing students were investigated and had completed the questionnaire, 49.4% of the participants were diagnosed to have acute gastroenteritis. The predominant symptoms were watery diarrhoea (90.8%) and abdominal cramps (71.3%). Of 28.9% of rectal swab isolates were identified as *Vibrio parahaemolyticus* O4:K55 (40.4%), *Salmonella* spp. (19.2%), *Vibrio cholerae* non O1/non O139/non O141 (11.5%), *Aeromonas trota* (3.9%), *Vibrio alginolyticus* (1.9%) and other co-infections (23.1%). The *tdh* gene was identified from all *V. parahaemolyticus* using multiplex PCR. The implicated food risk factor for gastroenteritis was boiled egg (adjusted prevalence rate ratio; PR=1.9, 95% CI, 1.04 – 3.79). However the bitter melon soup was not significantly associated for gastroenteritis (adjusted PR=1.3, 95% CI, 0.98 – 1.82). The population attributable fraction analysis indicated that boiled eggs item was an implicated food risk for this outbreak (PAF=45.8%). *Vibrio parahaemolyticus* O4:K55 was identified as a major causative agent for gastroenteritis in which the contaminated boiled eggs was a vehicle in this outbreak. Cross-contamination control should be emphasized in food operation plans among institutes.

INTRODUCTION

Gastroenteritis is one of the most common diseases worldwide. In developing countries with poor hygiene and sanitation, the prevalence of gastroenteritis was mainly due to enterobacteria such as *Escherichia coli*, *Salmonella* spp., *Shigella* spp., *Campylobacter jejuni* and *Vibrio cholerae*; as well as parasites such as *Entamoeba histolytica* and *Giardia intestinalis*, and some rotaviruses (Guerrant *et al.*, 1990). Salmonellosis infection was mostly caused through consumption of foods of animal origin such as beef, pork, poultry, milk, or egg-associated outbreak (CDC 1996a, 1996b). In Thailand, the incidence of enteric fever had been slowly decreasing over the past 10 years from 32/100,000 in 1984, to 28/100,000 persons in the population in 1993 (Suankratay *et al.*, 2001). However, the morbidity rates per 100,000 population per year of Salmonella Enteritidis infection

increased from 0.09 in 1990 to 1.47 in 1995 (Boriraj *et al.*, 1997). A recent study between 2004 and 2007 indicated the increase of human infections from several common serovars i.e., S. Stanley, S. Corvallis, and S. Choleraesuis in Thailand (Hendriksen *et al.*, 2009).

Additionally, a gram negative, halophillic bacterium, *Vibrio parahaemolyticus* was emerging as a causative agent of gastroenteritis in the US. (Tauxe, 1997) and worldwide (Yeung & Boor, 2004). Most outbreaks of *V. parahaemolyticus* gastroenteritis in USA, Japan, Taiwan and Thailand were associated with consumption of contaminated seafood (Yeung & Boor, 2004; McLaughlin *et al.*, 2005; Su & Liu, 2007). The number and severity of *Vibrio* spp. infected patients from non-toxigenic strains of *V. cholerae* and other non-cholerae *Vibrio* including *V. parahaemolyticus* and *Vibrio vulnificus* have increased, recently (Daniels *et al.*, 2000; Hanshoaworakul, 2005; Serichantalergs *et al.*, 2007). The enteropathogenic *V. parahaemolyticus* produced various virulence factors, including thermostable direct hemolysin (TDH), TDH-related hemolysin (TRH) and urease enzyme which was encoded by *tdh*, *trh* and *ure* genes, respectively (Honda *et al.*, 1991; Suthienkul *et al.*, 1995; Zhang & Austin, 2005). Previous studies demonstrated that *V. parahaemolyticus* gastroenteritis was associated with different combinations of O and K serotypes and possessed the tdh gene (Daniels *et al.*, 2000; Yeung & Boor, 2004; CDC, 2005) in which serotype O3:K6 caused several pandemic foodborne outbreaks (Altekruse *et al.*, 1997; Wong *et al.*, 2000; Harth *et al.*, 2009). In Thailand, several serovars of V. parahaemolyticus including, O3:K6, O1:KUT, O1:K25, O3:K46 O4:K68, O4:K9, and O4:K55 were isolated from symptomatic patients (Serichantalergs *et al.*, 2007).

On 13th September 2005, a cluster of acute diarrhoea among nursing students was observed in the emergency unit of a hospital. The investigation team conducted an outbreak investigation to determine the magnitude and associated risk factors of the gastroenteritis outbreak.

MATERIALS AND METHODS

The outbreak setting

On 13th September 2005, the Clinical Epidemiology Unit of Phramongkutklao Hospital was notified of an acute diarrhoea outbreak among a group of nursing students. The patients' experience were abdominal pain, vomiting and severe diarrhoea. An investigation was started on the afternoon of 13th September 2005 at the nursing college in Bangkok, Thailand.

Epidemiological investigation

A retrospective cohort study was conducted between 13th and 17th September 2005 to determine the risk factors for gastroenteritis. The study participants were 182 nursing students who stayed in the dormitory during the time of the outbreak, including 2nd, 3rd, and 4th year students. However the first year nursing students were on semester break and were outside the dormitory and therefore excluded from the study. A probable case definition was defined as the nursing students who lived in the dormitory and developed watery diarrhoea $\geq$ 2 times in a 24-hour period during 12th–15th September 2005 or had *V. parahaemolyticus*-positive isolates. Active cases were identified from amongst the study participants through the use of interviews and self-administered questionnaires. This questionnaire included demographic information, a history of food consumption and drinking water between 11th and 13th September 2005, clinical characteristics and medical treatment. Additionally, all study participants were requested to participate in screening for enteric bacterial infection using rectal swab cultures.

Environmental investigation

In the environmental investigation, food handlers, cooks, and food material suppliers were interviewed to obtain

information related to sources of raw food materials, food handling and storage practices, storage temperatures and food preparation processes. Food and water including the remaining raw food materials, drinking water, swab samples from chopping boards from the college canteen and private stalls outside the college and rectal swab cultures of the food handlers and cooks were obtained. The supplier stall in a marketplace was inspected for sanitation.

Identification of enteric pathogens

Rectal swab specimens were obtained from study participants and food handlers as well as environmental samples. All samples were determined as enteric bacterial pathogens including *Vibrio* spp., *Salmonella* spp., *Shigella* spp., *Campylobacter* spp., and *E. coli* at Clinical Microbiology Laboratory, the Phramongkutklao Hospital. The standard bacteriological identification methods were carried out by inoculation of the specimens onto MacConkey agar, *XLD agar* (Xylose lysine deoxycholate), TCBS agar (Thiosulfate citrate bile sucrose medium), Selenite broth, TSI agar (Triple sugar iron medium), and biochemical test (NCCLS, 2000).

All *Vibrio* spp. and *Salmolnella* spp. isolates were submitted for serotyping using slide agglutination sero-test antisera (S&A Reagents. Lab Ltd., Bangkok, *Thailand*) at the Reference Laboratory of the National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Thailand. The isolates identified as *V. parahaemolyticus* were tested for virulence gene markers, i.e., *tdh*, *trh* and *ure* using Christensen's urease testing and multiplex polymerase-chain-reaction (PCR) analysis (Bej *et al.*, 1999) as well as virulence genes of *V. cholerae* non O1 including cholera toxin (CT), zonula occludens toxin (ZOT), and accessory cholera enterotoxin were identified using the multiplex-PCR (Fields *et al.*, 1992; Shi *et al.*, 1998; Rivera *et al.*, 2001) at the reference laboratory.

Statistical analysis

The food-specific attack rate (AR) for illness was calculated. Chi-square test or Fisher's exact test and student t-test were performed. In order to estimate potential effect of various risk factors for gastroenteritis, we constructed a Poisson regression model with robust variance to obtain adjusted prevalence rate ratios (PRs) and 95% confidence intervals (CIs) (Barros & Hirakata, 2003; Zou, 2004). The criteria for selection variables into regression model was p-value less than or equal to 0.20 in order to control for confounding factors. The alpha levels of variables greater than 0.10 was removed using backward step-wise selection to produce the final model. The level of significant was set at two-sided p-value < 0.05. In this study, we estimated the population attributable fraction (PAF) of potential food risk factors for gastroenteritis (Brady, 1998). Data analysis was performed using software STATA version 9.0.

RESULTS

Epidemiological investigation

All of 182 female nursing students were investigated, however, 98.9% (180 of 182) completed self-administered questionnaires and were checked for enteric bacterial infection. We identified 89 (49.44%) students who had watery diarrhoea ≥ 2 times in a 24-hour period during 12^{th}–15^{th} September 2005 or had *V. parahaemolyticus* -positive isolates which met the case definition. The attack rates among cases for each year class of the students were 35.59%, 50.34%, and 53.23% in the second, third and fourth year nursing students, respectively. The mean age of the illness was 20.6 (SD = ±1.0) years old. The onset of the first symptomatic person who was infected with *V. parahaemolyticus* was started at 10.30 P.M. on 12^{th} September 2005. The highest peak of the outbreak occurred at 6.00 – 9.00 A.M. on 13^{th} September 2005 (Figure 1). The gastro-

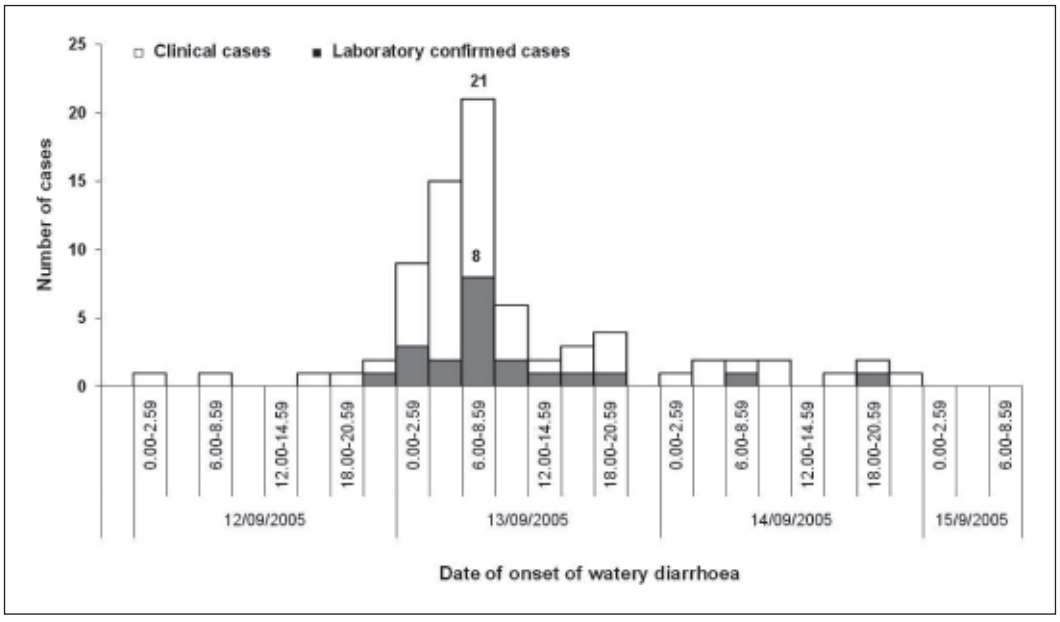

Figure 1. Cases of gastroenteritis infection in Nursing College, Bangkok, Thailand according to date of onset (September, 2005; N=77)

enteritis symptoms were watery diarrhoea (90.8%), abdominal cramps (71.3%), abdominal pain (59.8%), nausea/vomiting (36.8%), headache (25.3%), fever (20.7%), mucous diarrhoea (6.9%), sweating (6.9%), and bloody diarrhoea (1.1%). Twelve of 87 (12.6%) cases sought emergency medical care. In addition to the polymicrobial isolates, the proportion of those who were infected with *V. parahaemolyticus* was 80% (32 out of 40) while it was 10% (4 out of 40) of *Salmonella* spp. infection. When the study was focused on the participants who had *V. parahaemolyticus* -positive isolates, we found that 68.75% of participants (22 of 32) had suffered from watery diarrhoea while 31.25% (10 of 32) were asymptomatic.

The food specific attack rate for each food item was identified as shown in Table 1. In univariate analysis of food risk factors, the association of several food items including sweetened fried boiled egg, barbecue-pork with rice, sauce for barbecue-pork, boiled egg, and mixed sweet jelly were statistically significant with gastroenteritis in this outbreak. In multivariate analysis, we assessed the independent effect of food risk items for gastroenteritis and found that the significant independent food risk items for gastroenteritis was consumption of boiled eggs (adjusted prevalence rate ratio, PR=1.99, 95% CI, 1.04 – 3.79) while consumption of bitter melon soup was not statistically significant (adjusted PR=1.33, 95% CI, 0.98 – 1.82). From the final analysis, the other food risk items including sweetened fried boiled egg, barbecue-pork with rice, sauce for barbecue-pork, cucumber, mixed sweet jelly, Thai spicy paste, bitter melon soup and water from the canteen drinking cooler were not associated with this gastroenteritis outbreak. Boiled egg was a side dish to barbecue pork with rice served to all students at lunch on 12th September 2005. Additionally, the attack rate for nursing students who consumed boiled egg was 53.25% (82 of 154) while it was 26.92% (7 of 26) for those who did not consume the boiled egg. In addition, to focus on participants who had consumed the boiled egg and positive polymicrobial isolates, we found that 57.69% (30 of 52) of participants had *V. parahaemolyticus* -positive isolates, while 32.69% (17 of 52) had other bacterial-positive isolates.

Table 1. The analysis of food risk factors for gastroenteritis outbreak in a nursing college, Bangkok, Thailand (September, 2005)

Food risk item	Illness / Total (AR%)	Univariate analysis		Multivariate analysis; final model	
		p-value	Crude PR (95% CI)	*p*-value	Adjusted PR (95% CI)
11-Sep-05					
Chicken boiled-rice	7/15 (46.7)	0.840	0.95(0.54-1.66)		N/A
Noodle with barbecue pork	35/64 (54.7)	0.270	1.19(0.88-1.60)		N/A
Pork curry	47/87 (54.0)	0.206	1.21(0.90-1.64)		N/A
Sweeten fried boiled eggs	57/105 (54.3)	0.102	1.30(0.94-1.79)		–
12-Sep-05					
Fried bamboo-shoot	68/132 (51.5)	0.356	1.18(0.82-1.69)		N/A
Barbecue-pork with rice	85/163 (52.1)	0.025*	2.22(0.93-5.29)		–
Sauce for barbecue-pork	84/160 (52.5)	0.020*	2.10(0.97-4.55)		–
Black sauce	67/136 (49.3)	0.932	0.98(0.70-1.39)		N/A
Boiled eggs	82/154 (53.2)	0.013*	1.98(1.03-3.79)	0.037*	1.99(1.04-3.79)
Cucumber (1)	66/124 (53.2)	0.102	1.33(0.93-1.91)		–
Mixed Sweet jelly	84/160 (52.5)	0.020*	2.10(0.97-4.55)		–
Thai spicy paste	59/107 (55.14)	0.064	1.34(0.97-1.85)		–
Cucumber (2)	43/85 (50.6)	0.771	1.05(0.78-1.40)		N/A
Bitter melon soup	56/101 (55.8)	0.069	1.33(0.97-1.82)	0.067	1.33(0.98-1.82)
13-Sep-05					
Toast	59/118 (50.0)	0.837	1.03(0.76-1.42)		N/A
Fried eggs	59/116 (50.9)	0.609	1.09(0.79-1.49)		N/A
Sausage	56/114 (49.1)	0.909	0.98(0.72-1.33)		N/A
Fried noodles with pork sauce	67/131 (51.2)	0455	1.14(0.80-1.62)		N/A
KP drinking cooler	86/172 (50)	0.489	1.33(0.54-3.30)		N/A
Canteen drinking cooler	88/174 (50.6)	0.102	3.03(0.50-18.27)		–
Academic drinking cooler	34/79 (43)	0.209	0.79(0.58-1.08)		N/A

Note: * p-value < 0.05; AR= food-specific attack rate of exposure; CI= confidence interval; PR= Prevalence rate ratio; N/A= not available in the multivariate model.
The variables in multivariate model were sweeten fried boiled eggs, barbecue-pork with rice, sauce for barbecue-pork, boiled eggs, cucumber (1), mixed sweet jelly, Thai spicy paste, bitter melon soup and canteen drinking cooler .

To assess the relative importance of food risk factors for gastroenteritis in the final multivariate model, we calculated the population attributable fraction (PAF) among the study participants. The PAF of gastroenteritis attributable to boiled egg was 45.79% (95% CI, 10.23% – 67.26%) while it was 15.75% (95% CI, 3.01% – 26.82%) to bitter melon soup.

Environmental investigations

Almost all of the food served for students was cooked at the college canteen and only boiled eggs were delivered from supplier outside of the canteen. The preparation and handling of boiled eggs was investigated and it was found that approximately 200 boiled eggs were purchased from a supplier and served for lunch on 12th September 2005. During the environmental investigation outside of the college canteen, the shells were peeled from the boiled eggs and then placed into plastic bags, left in room temperature, and transported to the canteen on the following morning. At the college canteen, the boiled eggs without been re-heated or re-boiled were then served as a side dish to the barbecue pork with rice for lunch. This boiled eggs menu was the only ready-to-eat food item which was delivered together with other raw food materials from the supplier. Other items on the menu using eggs were cooked at the college canteen.

In the college canteen, no pathogenic bacteria in drinking water, on the chopping

board or others environmental swab were identified, as shown in Table 3. Nevertheless, it was found that fermented mussels and crabs served with papaya salad at a private food stall outside the college yielded *Vibrio alginolyticus* and *Vibrio furnissii*, respectively. Among food handlers of the college canteen, 10% (1 of 10) was *V. cholera* non O1/non O139/non O141 positive isolate and asymptomatic diarrhoea. From those interviewed, 90% (9 of 10) had no symptom of gastroenteritis and negative for polymicrobial isolates.

Laboratory investigation

A total of 180 rectal swabs from nursing students were examined. Fifty two of 180 (28.89%) samples showed positive isolates yielding *V. parahaemolyticus* O4:K55, *Salmonella* spp., *Aeromonas trota*, *V. cholerae* non O1/non O139/non O141, and other *Vibrio* spp., as shown in Table 2. Additionally, 9.62% (5 of 52) were co-infected with *V. parahaemolyticus* O4:K55 and *V. cholerae* non O1/non O139/non O141in this study. The multiplex PCR for virulence genes, *tdh* and *trh*, and analysis of urease enzyme revealed that all *V. parahaemolyticus* O4:K55 isolates were *tdh* gene positive, while *trh* gene and urease enzyme testing were negative. No virulence genes were identified from *V. cholerae* non O1/non O139/non O141 isolates.

DISCUSSION

An outbreak of acute gastroenteritis occurred among nursing students in Bangkok, Thailand, in which *V. parahaemolyticus* was identified as a major causative agent of the infection and related to consumption of boiled eggs. Of 49.44% of nursing students were identified as having a case of gastroenteritis. *V. parahaemolyticus* O4:K55 played an important role for this gastroenteritis outbreak. In the investigation, we found that the proportion of watery diarrhea among those who had *Vibrio parahaemolyticus* positive isolates was greater than *Salmonella*-positive isolates (78.95% *vs.* 23.68%). Focusing on bacterial isolation,

Table 2. The proportion of watery diarrhoea symptom among participants who had polymicrobial-positive isolates. (N=52)

Pathogens	Watery diarrhoea		
	Total n (%)	Asymptomatic n (%)	Symptomatic n (%)
Mono-infection			
V. parahaemolyticus O4:K55 (*tdh* gene positive)	21 (40.38)	6 (27.27)	15 (50.00)
V. cholera non O1/non O139/non O141	6 (11.54)	4 (18.18)	2 (6.67)
V. alginolyticus	1 (1.92)	1 (4.55)	–
Aeromonas trota	2 (3.85)	1 (4.55)	1 (3.33)
S. Braenderup	5 (9.62)	2 (9.09)	3 (10.00)
S. Corvalis	1 (1.92)	1 (4.55)	–
S. Montevideo	1 (1.92)	–	1 (3.33)
S. Stanley	3 (5.77)	3 (13.64)	–
Co-infection			
V. parahaemolyticus and *V. cholera non O1**	5 (9.62)	2 (9.09)	3 (10.00)
V. parahaemolyticus and *V. alginolyticus*	1 (1.92)	–	1 (3.33)
V. parahaemolyticus and *V. fluvialis*	1 (1.92)	–	1 (3.33)
V. parahaemolyticus and S. Anatum	1 (1.92)	–	1 (3.33)
V. parahaemolyticus and S. Stanley	1 (1.92)	1 (4.55)	–
V. parahaemolyticus and S. Braenderup	2 (3.85)	1 (4.55)	1 (3.33)
V. cholera non O1 and* S. Anatum	1 (1.92)	–	1 (3.33)

Note: * = *V. cholera* non O1/non O139/non O141

Table 3. The results of polymicrobial isolates in environmental investigation

Characteristics/Samples	Bacterial culture
Food handler (N=10)	
• Hand swab	negative
• Rectal swab	*V. cholera* non O1/non O139/non O141 (n =1)
Raw material in College canteen	negative
Raw material in Private stall	
• Fermented mussels	*V. alginolyticus*
• Fermented crabs	*V. furnisii*
Drinking water	negative
Environmental swab	
• Chopping boards	negative
• Others	negative

there was more likely to develop watery diarrhoea among those who had *V. parahaemolyticus* infection (50%) than *Salmonella* infection (13.3%). All severe watery diarrhoea participants who did not get antibiotic treatment by visiting emergency units were *V. parahaemolyticus* -positive isolates. Moreover, *V. parahaemolyticus* O4:K55 with the *tdh* gene (40.38%) was the most isolated pathogen in this outbreak. The pathogenic strain of *V. parahaemolyticus* O4:K55 in our investigation was similar with the recent studies that several serotypes of O3:K6, O4:K9, O4:K55, O4:K68, O1:KUT and O3:K46 with *tdh* gene were isolated from clinical patients in Thailand (Serichantalergs *et al.*, 2007; Wootipoom *et al.*, 2007).

The co-infection of *V. parahaemolyticus* O4:K55 and *V. cholerae* non O1/non O139/non O141 was observed. Of 60% of those who had co-infection presented watery diarrhoea and one of three suffered of the illness sought medical treatment at emergency unit. However, the frequency of watery diarrhoea symptoms of two vibrio co-infection was less than *V. parahaemolyticus* mono-infection (3 to 6 times *vs.* 1 to 10 times in 24-hour period), respectively. Given the limitation that the number of bacterial colonies was unable to be counted, the comparative severity attributable to mono-infection and co-infection of *Vibrio* spp. could not be determined.

This gastroenteritis outbreak was possibly contributed by contaminated boiled eggs which were consumed at lunch on 12th September 2005. Results of polymicrobial isolates showed that the proportion of bacterial isolates was greater in *V. parahaemolyticus* infection (93.75%) than others pathogens (85%) among participants who consumed boiled eggs. Subsequently the independent effect of those who consumed boiled eggs were a greater risk of developing gastroenteritis at 1.99 times (95% CI, 1.04 – 3.79) than those who did not consume boiled eggs. Consequently, the median incubation period of *V. parahaemolyticus* infection in this outbreak was 18.30 hours which corresponded with the established *V. parahaemolyticus* incubation period within 12 to 24 hours after exposure (Daniels *et al.*, 2000; Kaufman *et al.*, 2002).

The food item of boiled eggs was not a usual high risk food that caused *V. parahaemolyticus* outbreaks, in contrast to *Salmonella* spp. infection. However from our investigation including a food supplier interview, it was possible that boiled eggs were contaminated with bacteria through sewage, other raw foods, or ineffective cleansing of utensils and containers after

the shells were peeled off. Bacterial multiplication occurred during the long period of storage at room temperature for the following day delivery. The contamination after cooking was likely an indirect mechanism of infection. Several studies indicated that *V. parahaemolyticus* gastroenteritis was associated with consumption of improperly cooked seafood (Daniels *et al.*, 2000; Hanshoaworakul, 2005; McLaughlin *et al.*, 2005). The epidemiological investigation analysis and polymicrobial isolates supported that the food vehicle for gastroenteritis was strongly suggestive of a ready-to-eat boiled egg which could be from the food supplier's preparation procedure. This investigation also emphasized the importance of non-seafood food items that may be associated with the *V. parahaemolyticus* infection. Regarding cross contamination, the college's canteen should use fresh raw materials in preparation and avoid purchasing the ready-to-eat foods outside the canteen to prevent future foodborne gastroenteritis outbreaks. Additionally, supplier could avoid cross contamination with ready-to-eat food and awareness of improper food handling.

There were several limitations in this investigation. The possibility of the inadequate quality of self rectal-swab collection could have affected the bacteriology testing resulting in an underestimated rate of infection. In addition, an information bias could have been present by using the data collection method of self-administered questionnaires and the history of food consumption. It could be skewed by the relatively short recall period of young nursing students. Nevertheless, the questionnaire was designed to cover the components of the food menu to search for a true food vehicle and collected at the same time as the rectal swab cultures. As the food sources were no longer available for investigation, we could not determine the source of the etiologic pathogen. However, in the course of the environmental investigation, we could not found the true evidence of contaminated sewage in the cooking process used outside of the college canteen. We postulated that the hard-boiled eggs were possibly washed with water contaminated with sewage or other foods including raw seafood, raw eggs and other poultry, or there may have been ineffective cleansing of utensils and containers after the shells were peeled from the boiled eggs.

In summary, the findings were reported from an acute gastroenteritis outbreak in which *V. parahaemolyticus* O4:K55 was identified as a major causative agent for gastroenteritis in Thailand. In addition, we found a co-infection between *V. parahaemolyticus* O4:K55 and *V. cholerae* non O1/non O139/non O141 in this outbreak. Consuming contaminated boiled egg was a potential presumptive vehicle for acquiring the gastroenteritis. This resulted in the establishment of an effective food operation plan for the institute.

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