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ELISA for Seroepidemiological Study of Exposure to *Vibrio cholerae* of Population in Krabi Province, Thailand

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SUMMARY **Seroepidemiological study of *Vibrio cholerae* exposure was carried out in Krabi Province during January 1989 to December 1990 using indirect ELISA to determine serum antibodies to lipopolysaccharides (LPS) of *V. cholerae*. Among 363 serum samples obtained from cord blood and venous blood of healthy persons, aged from 6 months to over 50 years, 65% and 64% were found positive for specific IgG and IgM against LPS of *V. cholerae*, respectively. The seroprevalence of *V. cholerae* infection increased with age from that found at 6 months, being highest in the age groups of 30-49 years for IgG and 15-29 years for IgM. The seroprevalence of *V. cholerae* infection was higher among female Muslims and home-makers, and increased with the family income. The seroprevalence of cholera infection was also influenced by home location, methods of food storage and water supply. These data suggested that a large number of Krabi's population had *V. cholerae* infection.**

Diarrheal disease is a major health problem in developing countries. It is one of the top ten leading causes of mortality and morbidity in infants and older age groups.[1] Cholera, acute diarrhea, dysentery and food poisoning are found to be common diseases in Thailand.[2] Since 1984, cholera has been recorded in Krabi Province. When comparing the rate of cholera cases to the whole country, cholera case rates of Krabi Provine were higher than the other provinces during 1984-1988, and the rates ranged from 5.7-16.0 per 100,000 population.[3,4] Each reported case must be confirmed by isolation of *V. cholerae* from the patient's feces, and Krabi Hospital was the only place in the province that had this laboratory facility whereas other community hospitals and health centres in the villages did not. Therefore, total reported cholera cases could underestimate the real situation because they did not include those without stool culture confirmation, which may perhaps be the majority of cases.

In order to assess the magnitude of the problem, a serological test such as ELISA is commonly employed due to the fact that it is simple and suitable for mass screening in epidemiological work.[5,6] Once the person becomes infected, the immune response against the agent persists for a long period of time, even though the infectious agent itself has disappeared or is undetected. Therefore, antibody survey can indicate what has happened in the past in the community, reflecting period prevalence of infection. In this study, seroprevalence of *V. cholerae* infection in a healthy population in Krabi Province was studied using the indirect ELISA for detecting specific serum antibodies to LPS of this organism, allowing analysis of serological information in relation to demographic and possible risk factors.

MATERIALS AND METHODS

Data and sample collections

An apparently healthy groups of 323 people aged from 6 months to over 50 years, residents of Krabi Province, were randomly selected to enter the study with their consent, excluding those with a history of diarrhea within 6 months preceding the interview time. All subjects were interviewed concerning personal

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data such as age, religion, level of education, occupation, family income, eating habits and water supply. The parents of those below 15 years old were asked to give information about their children. All enrolled subjects were requested to give 5 ml of venous blood. The study also included 40 newborn babies delivered at Krabi Hospital, from whom 5 ml cord blood samples were collected at delivery and their mothers were interviewed for their family information. All subjects were classified into 10 age groups with a sex ratio of 1:1 (Table 1).

Blood specimens were allowed to clot at room temperature for 1 hour and centrifuged at 800 × g for 15 minutes. Sera were then collected and stored at -20°C until analyzed.

Table 1. Seroprevalence of *Vibrio cholerae* infection in various age groups.

Age group (years)	Number of Specimens	% of Seroprevalence (Mean level of OD ± SD)	
		IgG*	IgM**
Newborns	40	80 (0.36 ± 0.27)	0 (0.02 ± 0.01)
6 months	11	9 (0.12 ± 0.01)	27 (0.23 ± 0.18)
7–11 months	29	21 (0.14 ± 0.10)	45 (0.34 ± 0.28)
1–2	42	26 (0.18 ± 0.12)	69[b] (0.48 ± 0.18)
3–4	39	38 (0.22 ± 0.24)	79 (0.44 ± 0.18)
5–9	42	79[a] (0.31 ± 0.15)	88 (0.48 ± 0.16)
10–14	40	60 (0.31 ± 0.21)	81 (0.47 ± 0.24)
15–29	40	93[a] (0.52 ± 0.29)	90 (0.50 ± 0.16)
30–49	40	98 (0.55 ± 0.26)	70[b] (0.41 ± 0.18)
> 50	38	97 (0.49 ± 0.25)	57 (0.39 ± 0.23)
Total	363	65 (0.33 ± 0.26)	64 (0.38 ±0.23)

*Cut–off value for IgG was ⩾ 0.2 (calculated by Mean OD ± 3 SD of baby sera at six months of age)
**Cut–off value for IgM was ⩾ 0.3 (Calculated by mean of newborn sera)
a $P < 0.05$
b $P < 0.01$

Indirct enzyme-linked immunosorbent assay (ELISA)

Purified LPS of *V. cholerae,* El Tor O_{17} SR used as antigen was prepared according to the method of Chongsa-nguan *et al.*[7] The reference strain of *V. cholerae,* El Tor O_{17} SR was kindly provided by Dr. Wanpen Chaicumpa, Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol Univeristy, Bangkok.

Indirect ELISA was performed on serum samples for determining the seroprevalence of IgG anti-*V. cholerae* LPS. The test was modified from the methods of Chaicumpa *et al.*[8] and Chongsa-nguan *et al*[9] Flat-bottom plates (Greiner, Labor-technik, Germany) were coated with LPS (10 μg/ml) in 0.05 M carbonate buffer, pH 9.6 and incubated at 37°C overnight. The plates were washed three times with phosphate buffered saline containing 0.05% Tween 20 (PBS-T) to remove the unbound materials and 150 μl of PBS-T containing 0.5% bovine serum albumin (PBS-T-BSA) were added to each well to block the microtitre plates which were not occupied by the antigen. The plates were incubated in a humid container at 37°C for 1 hour. After washing three times with PBS-T, 100 μl of serum at dilution 1:200 were added to each well. Well with only PBS-T-BSA was used as a blank. Positive/negative serum control wells were included in the same plate. After one hour incubation at a 37°C, the plates were again washed three times with PBS-T. One hundred μl anti-human-IgG/IgM peroxidase (Dako Patts, Denmark) diluted 1:1,000 in PBS-T-BSA were added to each well. The plate was incubated at 37°C for 1 hour. After five washes with PBS-T, 100 μl of freshly prepared substrate solution (O-phenylenediamine. 2HCl) in citrate buffer (Abbott Laboratories, Chicago, IL, USA) were added to each well. The reaction was stopped after 30 minutes of incubation by

adding 50 μl of 1 N NaOH. Optical density (OD) was then measured using a spectrophotometer at 405 nm. To calculate the results, the blank OD value was subtracted from absorbance values of test samples. The OD value of the blank control was < 0.01. Cut-off values for positive reactions of IgG and IgM were $\geqslant 0.2$ and $\geqslant 0.3$, respectively.

Statistical analysis

The comparision of seroprevalence of *V. cholerae* among various age groups was calculated by Chi-square test. In order to compare the antibody levels, the statistical significance of means among various age groups was tested by one-way analysis of variance. Multiple comparisons of means of individual groups were done with the Newman-Keuls test, only if the overall F-test was significant. The distribution of each epidemiological characteristic between seropositive and seronegative groups of cholera was investigated by Chi-square test.

RESULTS

Among 363 Krabi healthy persons 236 (65%), and 233 (64%) persons had IgG and IgM antibodies to *V. cholerae,* respectively (Table 1). The means of IgG and IgM antibody levels to *V. cholerae* were 0.33 ± 0.26, and 0.38 ± 0.23, respectively.

Age distribution

The seroprevalence of IgG and IgM anti-LPS of *V. cholerae* in various age groups is shown in Table 1. It was found that the highest prevalence rates for IgG and IgM antibodies were in the age groups 30-49 years (98%) and 15-19 years (90%), respectively. The lowest prevalence for IgG was in those 6 months old (9%), and for IgM was in newborns (0%). Eighty percent of cord blood samples were seropositive (mean IgG level 0.36 ± 0.27) for IgG against *V. cholerae* and its level rapidly decreased with age, the lowest level being found

Table 2. The seroprevalence of IgG and IgM antibodies to *Vibrio cholerae* from healthy persons by various factors.

	Total N=363 (%)	No. (%) of seropositive for specific serum antibodies	
		IgG	IgM
Sex			
Male	182 (50)	120 (66)	106 (58)
Female	181 (50)	116 (64)	127 (70)
Religion			
Buddhism	169 (47)	128 (76)	95 (56)
Muslim	194 (53)	108 (56)	138 (71)
Occupation of family			
Agriculture	131 (36)	91 (70)	90 (69)
Employee	132 (36)	73 (55)	82 (62)
Business	40 (11)	29 (73)	28 (70)
Fishery	17 (5)	7(41)	8 (47)
Government service	24 (7)	18 (75)	10 (42)
Home–maker	19 (5)	18 (95)	15 (79)
Monthly income of family			
< 2,000	64 (18)	32 (50)	33 (52)
2,000–4,000	172 (47)	115 (67)	109 (63)
4,000–6,000	93 (26)	61 (67)	65 (70)
> 6,000	34 (9)	28 (82)	26 (77)
Education level (School) N=241*			
Not attending	21 (9)	21 (100)	13 (62)
Elementary	177 (73)	145 (82)	118 (67)
Secondary	24 (10)	21 (88)	16 (67)
Technical	14 (6)	14 (100)	7 (50)
University graduate	5 (2)	5 (100)	3 (60)
Home location			
Inland	190 (52)	134 (71)	113 (60)
Island or seashore	173 (48)	102 (59)	127 (73)
Type of food consumption			
Well–cooked	321 (88)	205 (64)	203 (63)
Half–cooked	42 (12)	31 (74)	30 (71)
Food storage			
Entirely covered	202 (56)	92 (46)	125 (62)
Note entirely covered	161 (44)	117 (73)	108 (67)
Eating behavior			
With spoon	260 (72)	175 (67)	171 (69)
With hand	14 (4)	11 (79)	7 (50)
Both	89 (24)	50 (56)	55 (62)
Water supply			
Tube well water	297 (82)	185 (62)	187 (63)
Canal water	7 (2)	4 (57)	4 (57)
Tap water	59 (16)	47 (80)	42 (71)

*Subjects over 5 to over 50 years old

at 6 months old children (0.12 ± 0.01). The IgG level then slowly increased for the next 5 years of life and rapidly increased after that point to reach its peak in the age group 30-49 years with a mean level of 0.55 ± 0.26. As for anti-LPS IgM, which was not discovered in all cord blood specimens, the seroprevalence significantly increased with age from 6 months old to the 15-29 years age group and gradually decreased in those above 30 years old.

Sex

The seroprevalence of IgG and IgM antibodies to *V. cholerae* LPS in relation to sex distribution is shown in Table 2. It was found that the prevalence of IgG and IgM antibodies in males was 66%, and 58% and in females was 64% and 70%, respectively. The seroprevalence of IgG antibody to *V. cholerae* in males was not significantly different from that in females. However, the seroprevalence of IgM antibody to *V. cholerae* in females was significantly higher than that in males ($p < 0.05$).

Religion

The religions in this population survey were Muslim (53%) and Buddhism (47%) (Table 2). The prevalence of IgG and IgM antibodies to *V. cholerae* LPS in Buddhists was 76% and 56%, while in Muslims it was 56% and 71%, respectively. The prevalence of IgG antibody to *V. cholerae* in Buddhists was significantly higher than that in Muslims ($p < 0.001$). In contrast, the prevalence of IgM antibody to *V. cholerae* in Muslims was significantly higher than that in Buddhists ($p < 0.05$). The seroprevalence was further examined in relation to religion and sex (Table 3). It revealed that the prevalences of both IgG and IgM in female Muslims (51% and 67%) was significantly higher than that in male Muslims ($p < 0.05$).

Occupation

Regarding occupations of studied population, they were categorized into 6 major groups namely; agriculture 36%, employee 36%, business 11%, government services 7%, fishery 5%, and home-maker 5% (Table 2). The seroprevalence of IgG and IgM antibodies to *V. cholerae* was highest among home-maker (95% and 79%, respectively).

Monthly income

The seroprevalence of IgG and IgM antibodies to *V. cholerae* increased with monthly income of family (Table 2). The higher the monthly income of the family, the higher was the seroprevalence of IgG and IgM antibodies to *V. cholerae.*

Education level

The children under 5 years old (122/363 or 34%) were excluded from the analysis regarding educational level. The seroprevalence of IgG (> 80%) and IgM (> 50%) antibodies to *V. cholerae* was high in all educational levels and not significantly different among them.

Home location

The seroprevalence of IgG and IgM antibodies to *V. cholerae* of individuals living inland was 71% and 60%, and of those living on islands or seashore was 59%, and 73%, respectively (Table 2). It was found that the prevalence of IgG antibody to *V. cholerae* of people living inland was significantly higher than that in those living on islands or seashore ($p < 0.05$). In contrast, the prevalence of IgM antibody to *V. cholerae* antigen of people living on islands or seashore was significantly higher than that of people living on inland ($p < 0.01$).

Eating habits

The family eating habits were classified into 3 categories : type of food consumption, food storage and eating behavior. It was found that majority of them (88%) consumed well-cooked food; 72% ate the food with a spoon, 24% used a spoon and sometimes the hand, very few used hand only; about 56% entirely covered food in storage and 44% did not. The seroprevalence of IgG and IgM antibodies to *V. cholerae* was above 50% regardless of any type of food consumption and eating behavior, with no significant difference among them ($P > 0.05$). However, the seroprevalence of IgG antibody to *V. cholerae* in people who had not entirely covered their food was significantly higher than in those entirely covered food ($p < 0.001$).

Water supply

In this population, people got their water supply from three sources;

Table 3. Seroprevalence of anti—*Vibrio cholerae* in Krabi healthy persons by religion and sex.

Religion	No. (%) of seropositive for specific serum antibodies			
	anti—*V. cholerae* LPS			
	IgG		IgM	
	Male	Female	Male	Female
Buddhism	71 (56)	57 (49)	53 (50)	42 (33)
Muslim	49 (41))	59 (51)	53 (50)	85 (67)
Total No.	120	116	106	127

tube well water (82%), tap water (16%) and canal water (2%) (Table 2). The seroprevalence rates of IgG and IgM antibodies to *V. cholerae* were more than 50% regardless of the type of water supply, with the highest seroprevalence in the group of using tap water (80% and 71%, respectively).

DISCUSSION

The indirect ELISA was performed to determine the levels of class specific antibodies to *V. cholerae* lipopolysaccharide (anti-LPS) and to estimate the seroprevalence among people who have been living in Krabi Province, Thailand. Measurable levels of IgG and IgM anti-LPS were found in individuals in all age groups, except IgM antibody was not detectable in newborns. The seroprevalence increased gradually with age. Of these, the highest seroprevalence as well as the highest mean level of IgG anti-LPS was found in the 30-40 years age group, while that of IgM anti-LPS was in the 15-29 years group. The seroprevalence among the studied population was higher than 50% and indicated that most areas of Krabi Province and possibly neighboring provinces [4] are endemic areas of *V. cholerae* infection (65% for IgG anti-LPS and 64% for IgM anti-LPS).

The level of IgG specific to LPS detected in most newborns could be due to maternal transfer which apparently disappeared at the age of 6 months. The IgG level significantly increased ($p < 0.05$) with age, suggesting that by 5 years of age the individuals in the area had experienced cholera infection (primary immune response). Subsequently upon reexposure to LPS antigen the individuals responded with a higher and persistent level of IgG (secondary immune response) as observed among people over 15 years of age. In contrast, the level of IgM anti-LPS of infants was negative at birth, and subsequently responded 6 months later, then significantly increased during the 1-2 years period, increased markedly after that till age 30, then started to decrease. These results were similar to those previously found in another study. [9]

The seroprevalence of IgM antibodies to *V. cholerae* was particularly high in female Muslims (Table 3). The prevalence of both IgG and IgM antibodies to *V. cholerae* were markedly higher among home-makers when compared with other occupations. This higher prevalence might possibly be due to the greater chance of re-exposure to *V. cholerae* contaminated food or water in their daily lives, particularly those who live in rural areas as previously reported. [6,10] In addition, the exposure of females to cholera infection could also occur via their contaminated hands while cleaning the children's bodies after defecation. [6] Regarding religion, the exact causes of these differences are still unknown and should be further investigated. However, one explanation might be that the people in each religion have their own behaviors and habits in consuming certain foods which might alter the physiological conditions or pH in the stomach leading to their unequal degrees of susceptibility to the organisms. [11] Nevertheless, cholera is generally transmitted by the fecal-oral route, from person to person, and through close contact under unhygienic and overcrowded conditions. [12,13] Therefore, mass gathering of people for some religious activities might possibly be another factor that could cause the individuals to be exposed to infection. [13]

The seroprevalence of *V.cholerae* found in this study was significantly increased with educational levels and family monthly incomes. These results might reflect the rapid economic expansion in Krabi Province during the past few years, due to tourism and the palm oil industry, while health education and sanitation of people in such community was not improved together with the rapid economic changes. It seemed that the educational level or their knowledge might have not affected their attitudes and practices especially on personal hygiene and health education.

The prevalence rate of IgM antibody was significantly higher among the people who lived near the seashore than among those living inland ($p < 0.01$). One reason which might be given for this finding is the fact that there is higher migration of non-immune individuals from the Northeast, Thailand to the seashore area of Krabi Province (to serve as fishery labours) than those to inland area. Primary infection of these migrant populations resulted in IgM antibody response. The people who lived inland are the more permanent residents in the area and hence recurrent infections would expectedly give rise to the relatively higher IgG isotypes of antibodies demonstrated among them. In general, poor water supply, [13-15] overcrowded living conditions, [12,13] and unsanitary food handling and storage [16,17] are important risk factors in cholera infection. Adult females, mothers or homemakers are the high risk groups due to association with *V. cholerae* contaminated food and water.

Since cholera infection is still a public health problem of the community, these results suggest that diarrhea control measures among this population should be reevaluated. Efficient and effective health education concerning personal hygiene which is appropriate to each locality and their culture, sanitation, and improvement of water systems should be emphasized.

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