

Dot-ELISA for Seroepidemiological Study of Exposure to *Shigella flexneri*

Orasa Suthienkul¹, Anong Poomchart¹, Uraivan Kositanont²,
Kanokrat Sirpanichgon¹ and Kanda Vathanophas¹

Diarrheal disease is one of the top ten leading causes of morbidity and mortality in infants and older age groups in Thailand.¹ Etiologic agents in adult diarrheic patients are shigellae and vibrios, whereas shigellae and *Escherichia coli* are more common in children.² In one study *Shigella* was isolated from 13% of Thai children under 5 years with diarrhea and 0.2% of those without diarrhea.³ *Shigella* spp. were isolated from 44% of Thai children with mucoid or bloody feces.⁴ *S. flexneri* was the predominant serogroup causing shigellosis, followed by *S. sonnei*.⁵ Since 1984, shigellosis has been recorded in Krabi Province. The case incidence was higher than in other provinces with rates of 84.3 and 57.0 per 100,000 population in 1987 and 1988, respectively.^{5,6} Due to lack of laboratory facilities in community hospitals and health centres in the villages, most shigellosis cases reported were clinically diagnosed without stool culture confirmation. Therefore, the total shigellosis cases reported could be underestimating the real situation.

For more efficient prevention

SUMMARY A seroepidemiological study for determining serum antibodies to lipopolysaccharides (LPS) of *Shigella flexneri* using dot-ELISA was carried out in Krabi Province, Thailand, from January 1989 to December 1990. From 363 serum samples obtained from cord blood and from venous blood of healthy persons aged from 6 months to over 50 years, 56% and 22%, respectively, were found to be positive for specific IgG and IgM antibodies to *S. flexneri* LPS. The IgG prevalence was initially detected at 3-4 years of age and then rose sharply with age. In contrast, IgM was detectable earlier, with much lower prevalence than that of IgG. The highest seroprevalence values were in the age groups 30-49 years for IgG and 15-19 years for IgM. The seroprevalence of *S. flexneri* infection was statistically higher among males, Buddhists, businessmen, and those with elementary education. *S. flexneri* infection was not associated with family income, home location, eating behaviour or water supply. These seroepidemiologic data demonstrated that most of the population in Krabi Province had been infected with *S. flexneri*.

and control of shigellae infection, epidemiological aspects of the disease need to be further investigated. Antibody survey can indicate what has happened in the past in the community, reflecting period prevalence of the infection. A serological test such as Dot-ELISA is simple, rapid, and suitable for mass screening in epidemiological work.⁷⁻⁹ In addition, a positive reaction can be visualized (as a colored spot) by the naked eye, thus making it a portable immunological technique that can be used in field studies. Thus, seroprevalence of *S. flexneri* infec-

tion in a healthy population in Krabi Province was determined using a Dot-ELISA for detecting specific serum antibodies to lipopolysaccharide (LPS) of *S. flexneri*; its relationship to general health factors of the population were also investigated.

From the ¹Department of Microbiology, Faculty of Public Health, ²Department of Microbiology, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand.

Correspondence : Orasa Suthienkul

MATERIALS AND METHODS

Study population

A total of 363 apparently healthy persons living in various districts of Krabi Province, aged from 6 months to over 50 years, together with newborn babies delivered at Krabi Hospital, were randomly selected for entry into this study during the period from January 1989 to December 1990. The subjects had no history of diarrhea or dysentery within 6 months preceding the study and were interviewed concerning their personal data such as sex, age, religion, level of education, occupation, family income, home location, eating habits and house water supply. The information concerning the newborn babies and children (< 15 years) were given by their parents. The subjects were classified into 10 age groups (Table 1).

Five millilitres of venous blood samples were collected from each enrolled subjects, and fetal cord blood samples were collected at delivery. All blood specimens were allowed to clot at room temperature for one hour and centrifuged at $800 \times g$ for 15 minutes. Sera were stored at -20°C until tested.

Dot-enzyme-linked immunosorbent assay (Dot-ELISA)

Purified LPS of *S. flexneri* serotype Y used as antigen for coating onto nitrocellulose membrane (NCM) discs was kindly provided by Dr J Edward Brown, Armed Forces Research Institute of Medical Sciences, Bangkok.

Dot-ELISA was performed on serum samples to determine the seroprevalence of IgG and IgM anti-*S. flexneri* LPS. The test was modified from the method of Beutin *et al.*⁹

NCM discs (5 mm diameter, pore size $0.45 \mu\text{m}$; Schleicher and Schuell, USA) were transferred into each well of a Costar plate. Purified

Table 1. Seroprevalence of *Shigella flexneri* infection in various age groups of healthy persons.

Age groups (years)	Number of specimens	% Seroprevalence (Mean value of titre \pm SD)	
		IgG*	IgM**
Newborns	40	75 (1.84 \pm 0.84)	0 (0.98 \pm 0.22)
6 months	11	9 (0.93 \pm 0.01)	0 (1.70 \pm 0.51)
7-11 months	29	10 (0.94 \pm 0.13)	21 (1.90 \pm 0.54)
1-2	42	10 (1.01 \pm 0.40)	21 (2.00 \pm 0.54)
3-4	39	36 ^a (1.37 \pm 0.75)	33 (2.05 \pm 0.61)
5-9	42	55 (1.54 \pm 0.83)	26 (2.04 \pm 0.53)
10-14	42	76 ^a (1.89 \pm 0.98)	36 (2.24 \pm 0.51)
15-29	40	85 (2.69 \pm 1.03)	45 (2.23 \pm 0.63)
30-49	40	90 (2.71 \pm 0.87)	15 ^b (1.85 \pm 0.56)
> 50	38	68 (2.07 \pm 1.00)	5 (1.45 \pm 0.58)
Total	363	56 (1.77 \pm 1.00)	22 (1.86 \pm 0.65)

* Cut-off titre for IgG was 1:16 (calculated by mean \pm 3 S.D. of baby sera at six months of age)

** Cut-off titre for IgM was 1:512 (background value in cord blood of newborn)

a $P < 0.05$

b $P < 0.01$

LPS of *S. flexneri* was diluted 20 $\mu\text{g}/\text{ml}$ in 0.05 M carbonate buffer, pH 9.6 and 0.5 μl of the diluted antigen were spotted on each NCM disc using a micropipette. The loaded discs were air-dried overnight at room temperature. After desiccation in the oven at 80°C for 2 hours, 200 μl of filler solution⁹ were added into each well and in-

cubated at 37°C for 1 hour. After removing the filler solution, the discs were washed once with 0.05 M NaCl containing 0.05% Tween-20 (NaCl-T). Serum specimens were serially diluted four-fold in filler solution and 100 μl of the diluted samples were added to each well containing the antigen sensitized discs in duplicate. Filler solution

was added instead of serum to a blank well. A positive serum control against LPS of *S. flexneri* ($\geq 1:512$) and negative serum controls ($< 1:16$) were included in each assay. The plates were incubated at 37°C for 1 hour and were washed with NaCl-T three times at 5 minute intervals. One hundred microlitres of anti-human-IgG/IgM alkaline phosphatase (Kirkegaard and Perry Laboratories, Inc, Gaithersburg, MA, USA) diluted 1:500 in filler solution were added to each well and incubated at 37°C for 1 hour. After three washes with NaCl-T at 5 minute intervals, 100 μ l of freshly prepared substrate solutions of naphthol AS-MX phosphate (0.4 mg/ml, Sigma Chemical Company, MO, USA) and Fast Red TR salt (6 mg/ml, Sigma Chemical Company, MO, USA) in 0.2 M Tris, pH 8.0 containing 2 mM MgCl₂ were added to each well and incubated at room temperature for 30 minutes. The color reaction was terminated by washing the discs three times with distilled water.

Discs with positive reactions showed the distinct red-pink spots on the labelled sites whereas discs with negative reactions showed no color on the spotted sites. Titres were determined as the highest dilution which showed positive results. Cut-off titres for positive reactions of IgG and IgM were 1:16 and 1:512, respectively.

Statistical analysis

Reciprocal titres of antibodies to *S. flexneri* LPS were transformed to their logarithmic values before determination. The comparison of seroprevalence of *S. flexneri* among various age groups were tested statistically using a 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 epidemiologic characteristic between seropositive and seronegative groups of shigellosis was investigated by Chi-square test.

RESULTS

The seroprevalence of 363 healthy persons was 56% (203/363) for IgG and 22% (79/363) for IgM antibodies to *S. flexneri* (Table 1). The means of IgG and IgM antibody levels to *S. flexneri* were 1.77 and 1.86, respectively.

Age distribution

The seroprevalence of IgG and IgM anti-LPS of *S. flexneri* in various age groups is shown in Table 1. It was found that the highest prevalence rates for IgG and IgM antibodies to *S. flexneri* were in the age groups of 30-49 years (90%) and 15-29 years (45%), respectively. The lowest prevalence for IgG was in those aged 6 months (9%), and for IgM was in newborns (0%), and 6 months (0%). Cord blood samples were seropositive (mean IgG level 1.84 ± 0.84) for IgG against *S. flexneri* in 75%; the level rapidly decreased after birth, the lowest level (9%) being found at age 6 months (0.93 ± 0.01). The IgG level then rapidly increased with age to reach the highest seroprevalence rate (2.71 ± 0.87) in the 30-49 years age group. All newborns and 6 months old children were seronegative for IgM against *S. flexneri*. However, the seroprevalence of IgM antibody to *S. flexneri* was much lower than the IgG prevalence, which slightly increased with age and then significantly dropped in those above 30 years old.

Sex

The sex ratio of the subjects in this study was approximately 1:1 (Table 2). It was found that the prevalence of IgG and IgM antibodies in males were 58% and 16%

and in females were 54% and 28%, respectively. The prevalence of IgM antibody to *S. flexneri* in females was significantly higher than that in males ($p < 0.01$).

Religion

The religions in this studied population were Buddhism (47%) and Muslim (53%) (Table 2). The prevalence of IgG and IgM antibodies to *S. flexneri* LPS in Buddhists were 66% and 20%, and in Muslims were 47% and 24%, respectively. The prevalence of IgG antibody to *S. flexneri* in Buddhists was significantly higher than that in Muslims ($p < 0.01$). In addition, the seroprevalence was further investigated in relation to religion and sex (Table 3). It was found that the prevalence of both IgG and IgM antibodies in male Buddhists was higher than that in female Buddhists, whereas prevalence of IgG and IgM in female Muslims was higher than that in male Muslims. There was a significant difference only in the IgG prevalence according to sex and religion characteristics ($p < 0.05$).

Occupation

The occupations of the family in this survey population were agriculture 36%, labourer 36%, business 11%, government service 7%, home-maker 5%, and fishery 5% (Table 2). The highest seroprevalence of IgG and IgM antibodies (75% and 35%, respectively) to *S. flexneri* LPS was found among the business group. The seroprevalence of IgG antibody in the business group was significantly higher than that in other occupations ($p < 0.05$).

Monthly income

Monthly income of families was classified into 4 groups (Table 2). Most people (47%) had incomes of 2,000-4,000 baht/month whereas of the others 26% had monthly incomes of 4,000-6,000 baht, 18% had less than 2,000 baht and 9% had more than 6,000 baht. The

Table 2. Seroprevalence of IgG and IgM antibodies to *Shigella flexneri* LPS from healthy persons by various factors.

Factors	Total N = 363 (%)	No (%) seropositive for specific serum antibodies	
		IgG	IgM
Sex			
Male	182(50)	105(58)	29(16)
Female	181(50)	98(54)	50(28)
Religion			
Buddhism	169(47)	111(66)	33(20)
Muslim	194(53)	92(47)	46(24)
Occupation of family			
Agriculture	131(36)	71(54)	29(22)
Labourer	132(36)	64(49)	25(19)
Business	40(11)	30(75)	14(35)
Government service	24(7)	16(67)	3(13)
Home-maker	19(5)	13(68)	4(21)
Fishery	17(5)	9(53)	4(24)
Monthly income of family (baht)			
< 2,000	64(18)	30(47)	10(16)
2,000-4,000	172(47)	95(55)	36(21)
4,000-6,000	93(26)	57(61)	25(27)
>6,000	34(9)	21(62)	8(24)
Educational level (school) N = 241*			
Not attending	21(9)	13(62)	5(24)
Elementary	177(73)	132(75)	33(19)
Secondary	24(10)	19(79)	10(42)
Technical	14(6)	11(79)	3(21)
University	5(2)	5(100)	1(20)
Home location			
Inland	190(52)	116(61)	39(21)
Island or seashore	173(48)	87(50)	48(28)
Type of food consumption			
Well-cooked	321(88)	178(55)	68(21)
Half-cooked	42(12)	25(60)	11(26)
Food storage			
Entirely covered	202(56)	118(58)	45(22)
Not entirely covered	161(44)	185(53)	34(21)
Eating behaviour			
With spoon	260(72)	154(59)	55(21)
With hand	14(4)	8(57)	2(14)
Both	89(24)	41(46)	22(25)
Water supply			
Tube well water	297(82)	159(54)	68(23)
Canal-water	7(2)	5(71)	1(14)
Tap water	59(16)	38(64)	16(27)

* Subjects 5 to 50 years old

seroprevalence of IgG and IgM antibodies to *S. flexneri* increased with monthly income of family but were not significantly different among them.

Educational level

The children under 5 years old (34%) were excluded from the analysis regarding educational level. Then the subjects were classified into 5 groups : elementary school (73%), secondary school (10%), not attending school (9%), technical school (6%) and university (2%) (Table 2). The seroprevalence of IgG and IgM antibodies to *S. flexneri* LPS was not correlated with the educational levels. However, the seroprevalence of IgG antibody in both sexes who had elementary education was statistically higher than those in both sexes who had other education, but a significantly high seroprevalence of IgM antibody was found only in females ($p < 0.01$) (Table 4).

Home location

From the results of this survey, the people living on the mainland constituted 52% and the rest, living on islands or along the seashore, comprised 48% (Table 2). The seroprevalence of IgG and IgM antibodies to *S. flexneri* of people living on the mainland were 1% and 21%, and those living on the islands or along the seashore were 50% and 28%, respectively. There was no relation between the seroprevalence of IgG and IgM antibodies to *S. flexneri* and home location ($p > 0.05$).

Eating habits

The family eating habits were divided into 3 categories : type of food consumption, food storage and eating behaviour (Table 2). It was found that the majority of them (88%) consumed well-cooked food; about 56% practiced proper food storage, while 44% did not have a proper food storage; 72% ate food

Table 3. Seroprevalence of anti-*Shigella flexneri* LPS in healthy persons by religion and sex.

Religion	No (%) seropositive for specific serum antibodies (anti- <i>S. flexneri</i>)			
	IgG		IgM	
	Male	Female	Male	Female
Buddhism	65(62)	46(47)	16(55)	17(34)
Muslim	40(38)	52(53)	13(45)	33(66)
Total	105	98	29	50

Table 4. Seroprevalence of anti-*Shigella flexneri* LPS in healthy persons by education levels and sex.

Education levels	No (%) seropositive for specific serum antibodies (anti- <i>S. flexneri</i>)			
	IgG		IgM	
	Male	Female	Male	Female
Not attending	4(4)	9(10)	3(16)	2(6)
Elementary	68(73)	64(74)	6(32)	27(82)
Secondary	12(13)	7(8)	8(42)	2(6)
Technical	5(5)	6(7)	2(11)	1(3)
University	4(4)	1(1)	0(0)	1(3)
Total	93	87	19	33

with spoons, 24% used spoons and with hands sometimes; very few used hands only. The seroprevalence of IgG antibody to *S. flexneri* LPS in all groups was higher than that of IgM antibody, however there were no significant differences among them ($p > 0.05$).

Water supply

In this population, water supplies were from three sources: tube well water (82%), tap water (16%) and canals (2%) (Table 2). The seroprevalence of IgG antibody to *S. flexneri* LPS was higher than that of IgM regardless of types of water

supply with the highest seroprevalence (71%) in the group using canal water.

DISCUSSION

Shigellosis has been one of the major public health problems in Krabi Province. The case rates in some years were even higher than in other areas of the country.^{5,6} The reported shigellosis cases compiled by the Division of Epidemiology, Ministry of Public Health, Thailand, indicated that *S. flexneri* 2a was the most common causative serotype and occurred most fre-

quently in the 0-4 age group with no difference in sex.⁵ It was found that all *S. flexneri* bacteria, except serotype 6, have a basic O-antigen structure. This basic structure is found in *S. flexneri* serotype Y.¹⁰⁻¹² Accordingly purified LPS of *S. flexneri* serotype Y was used as antigen for determination of *S. flexneri* antibodies in this study.

This study revealed that the children from birth to 6 months old still had maternal IgG anti-LPS of *S. flexneri*, and were seronegative for IgM antibody due to low infection at this age groups and the inability of IgM class to cross the placenta.¹³⁻¹⁵ Together with the common practice of breast-feeding, this could explain the lowest prevalence of evidence of shigella infection reported in children below 6 months old. However, as the children get older, receive supplementary food and increase their activities, the chances of shigella exposure are also increased.¹⁶ Antibody titres against the serotype Y of *S. flexneri* were previously reported to be always found after natural and experimental infection.^{11,12} It appeared that the study children began having *S. flexneri* infection not earlier than 7 months of their lives. If the children under 6 months old were excluded, 55% (172/312) of the study population were seropositive for IgG antibodies to *S. flexneri*, so that about half of this population had *Shigella* infection at sometime and could be a potential source of *Shigella* in the community, especially in areas with poor sanitation.¹⁷

The highest prevalence and mean levels (\bar{X}) of IgG and IgM antibodies to *S. flexneri* LPS were in the 30-49 years (90%, $\bar{X} = 2.71$) and 15-29 years (45%, $\bar{X} = 2.23$) age groups, respectively. These results were similar to those previously found in other studies.^{18,19} These anti-*S. flexneri* LPS levels might result from a previous *S. flexneri* infection.¹⁸ In fact, a majority of shigella infections may

be subclinical.¹⁸ None of the individuals interviewed had a history of diarrhea or dysentery within 6 months preceding the study. Further study eg faecal cultures should confirm carrier states of *S. flexneri* in this population.

The seroprevalence of IgG antibody to *S. flexneri* LPS was related to sex, occupation and religion. The prevalence was high among males, Buddhists, and businessmen. It appeared that in early life the attack rate of shigellosis was higher in males than females, but after about 20 years of age the incidence was reversed.²⁰ The seroprevalence of IgG antibodies in males and females in this study showed no significant differences. However, the seroprevalence of IgM antibody in females was significant higher than that in males. The high prevalence found in businessmen might possibly reflect susceptibility to *Shigella* infection through consuming contaminated food or water from restaurants or food vendors. It is known that as small a dose as 10 organisms is sufficient to cause infection.²¹ Regarding religion, the cause of these differences is still unknown. It is conceivable that some activities of religious events in the temples associated with food offering might the individuals to be exposed to the infection.

The seroprevalence of *Shigella* was associated with education and sex. There was a high prevalence in lower educated females, and in males, which might be related to poverty, poor personal hygiene, poor nutritional status, and crowded living condition.¹⁷ The transmission of shigellosis is reported to be associated with poverty and overcrowding.²² In this study, home location was not significantly associated with *S. flexneri* seroprevalence. However, there was a high prevalence in those living inland which might be due to overcrowded living conditions.²²

Shigella infection is reported to be more often attributable to eating of raw or ready cooked food which was liable to contamination from fingers or by flies.²⁰ Regarding the type of food consumption, food storage, eating behaviour, and types of water supply, there was no association with *Shigella* seroprevalence in this study. It was not known whether the study population having more or less similar eating behaviour. Addition qualitative data concerning eating behaviour would help in defining the common modes of transmission in this population. These include clinical surveillance, improving personal and domestic hygiene, water supplies, and sanitation to prevent *Shigella* infection.

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