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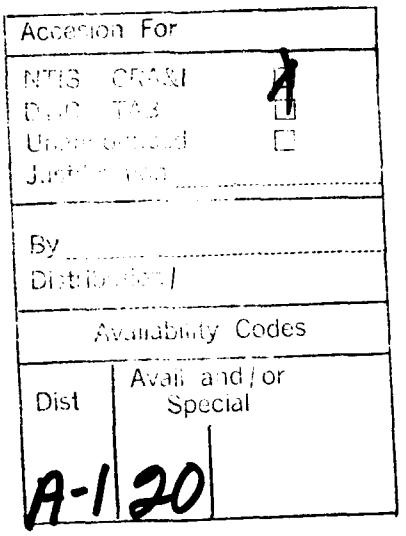







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# *Campylobacter* Immunity and Quantitative Excretion Rates in Thai Children

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*Campylobacter* species were isolated from 61 (15%) of 416 Thai children <5 years old with diarrhea. Although the baseline levels of *Campylobacter*-specific antibody increased with age, 80.3% of *Campylobacter*-infected children seroconverted compared with 12.9% of 45 *Shigella*-infected patients used as controls. The response to acute infection was greatest in the 6- to 12-month-old group. Nonseroconverters had higher initial IgG levels than did seroconverters ($P = .001$). Quantitative cultures showed a range of 1–8 $\log_{10}$ *Campylobacter* cfu/g of stool (median, 6.0 $\log_{10}$), and the seroconversion rate was highest in those with the highest *Campylobacter* excretion. Fecal *Campylobacter* excretion was inversely related to age ($\chi^2$ for trend, $P = .03$). These studies indicate that endemic *Campylobacter* exposure frequently induces seroconversion in young children, whether *Campylobacter* is isolated as a single pathogen or one of multiple pathogens, and that fecal excretion of the organism is inversely related to the age-related immune response to infection.

In developing countries such as Thailand, infection with *Campylobacter* species is highest in children <1 year of age, and the case-to-infection ratio falls with age [1], a pattern found in other developing areas [2]. Because most *Campylobacter* infections occurring in persons >2 years of age in such populations are asymptomatic, it is likely that disease-specific immunity develops during early childhood [3].

Recent studies have indicated that the expression of illness after *Campylobacter* infection is influenced by both strain characteristics and preexisting immunity [1–3]. However, the role of *Campylobacter* species in polymicrobial infections is unclear. In vitro studies suggest that other pathogens, including *Shigella* and *Salmonella* species and *Escherichia coli*, can enhance invasiveness of *Campylobacter jejuni* [4]. Furthermore, the finding of *Campylobacter* infection in asymptomatic subjects suggests that under some circumstances *Campylobacter* may be nonpathogenic [5]. To demonstrate how primary and secondary infections induce the development of protective immunity, we investigated the immune response in the case of polymicrobial infection in which *Campylobacter* and another enteropathogen were isolated and the relationship between intensity and duration of infection as manifested by quantitative stool load and immunity.

## Materials and Methods

*Patient population.* From 1 July to 31 December 1987, we studied children <5 years old who presented to the outpatient department at Children's Hospital (Bangkok) with acute diarrhea (defined as three or more loose stools in 24 h or one loose stool plus fever, vomiting, or abdominal pain). Patients were excluded if the diarrhea had lasted >3 days or if the patient had recently received antibiotics. Age, symptoms, and clinical and treatment data were recorded, and stool and serum specimens were collected. Any patient with a positive stool culture for *Campylobacter* or *Shigella* species was visited at home 2 and 4 weeks later for collection of clinical data and stool and serum specimens. Statistical analysis was done by the *t* test, $\chi^2$, or Fisher's exact test, using SAS (SAS Institute, Cary, NC) and Abstat (Anderson-Bell, Canon City, CO) software.

*Microbiologic protocol.* Stool specimens were plated onto MacConkey and Hektoen enteric agar (Difco, Detroit) before and after enrichment in Selenite-F enrichment broth (Becton Dickinson Microbiology Systems, Cockeysville, MD) as previously described [6]. *E. coli* were processed by DNA hybridization assays [7]. *Cryptosporidium* species, *Giardia* and amoeba cysts, and helminthic ova were detected as previously described [7]. Rotavirus was identified by monoclonal antibody ELISA [7]. Stool white and red blood cells were quantitated by direct microscopy.

*Campylobacter* species were isolated using the membrane filtration technique [8] or an antibiotic-containing *Campylobacter*-selective enrichment medium [9] followed by subculturing using the membrane filter technique after overnight microaerobic incubation. *Campylobacter* isolates were confirmed by standard procedure, identified to the species level us-

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ing hippurate hydrolysis, and serotyped according to the Lior system [10]. Quantitative cultures were done whenever *Campylobacter* species were isolated, using a standard dilution technique as follows: 1 g of stool was suspended into 1 mL of sterile normal saline, and then five serial 10-fold dilutions were done; 0.1 mL from each dilution was spotted into 0.45-μm sterile membrane filters (Millipore, Bedford, MA) that previously had been placed on *Brucella* agar containing sheep blood without antibiotics [8]. After incubation as above, colonies were counted to determine the number of colony-forming units per gram of stool, and results were rounded to the nearest $\log_{10}$ integer.

*Serology.* Sera, stored at −20°C on the day of collection, were tested in an ELISA using *C. jejuni* group-specific surface proteins as the antigen and immunoglobulin class-specific peroxidase conjugates as previously described [11]. Sera were assayed at single screening dilutions, 1:50 for IgA, 1:100 for IgG, and 1:200 for IgM.

## Results

*Epidemiology of Campylobacter infection.* A total of 416 children (56% boys) with acute diarrheal illnesses were enrolled in the study. Their mean age was 15.1 months (1SD, 8.9). *Campylobacter* species were isolated from 18.8% of children <12 months of age, 12.3% of those 12–23 months, and 10.3% of those 24–59 months. The age-related decrease in isolation rate was statistically significant ($P$ = .04). The isolation rates were similar in boys and girls.

*Microbiology. Shigella* species were isolated from 20% and *Campylobacter* species from 15% of 416 children with diarrhea. Mixed infections involving two or more enteric pathogens were observed in 91 children.

*Clinical features of Campylobacter infections.* Children were divided into three age groups (<12, 12–23, and 24–59 months). Among 37 patients infected with only *Campylobacter* species, the proportion with watery stools rose (from 21% to 40%) and the proportion with blood and mucus in stools fell (from 31% to 0) with increasing age. The age-related decrease in bloody stools was significant ($P$ = .02). In comparison, among the children not infected with *Campylobacter* species, no such trends were found. For none of the other clinical features were any age-related trends noted.

*Features of sole Campylobacter infections and mixed infection.* Of the patients from whom *Campylobacter* species were isolated, 37 (60.7%) had only that pathogen identified, while 24 (39.3%) had mixed infections. *C. jejuni* was isolated from 29 patients as the sole organism and from 15 patients concomitantly with another pathogen. *Campylobacter coli* was isolated from 4 patients as the sole pathogen and from 3 patients as part of a mixed infection. Nine isolates were group 2 aerotolerant *Campylobacter* species (now referred to as *Arcobacter butzleri* [12]) and 1 was *Campylobacter upsaliensis* [13]. *Shigella* species were the most common pathogen identified in this study, but only one coinfection with *Campylobacter* species was recognized, probably because most *Campylobacter* infections involved children <2 years whereas the opposite was true for *Shigella* species.

The 63 *Campylobacter* isolates from 61 patients at the initial visit included 44 *C. jejuni* strains represented by nine Lior serotypes; in addition, 5 *C. jejuni* strains were untypeable. The six most common Lior serotypes were 9 (14%), 4 (14%), 19 (14%), 11 (11%), and 28 (11%). In addition, 9 isolates of *C. coli* were identified, representing seven Lior serotype groups. This degree of heterogeneity has been observed in previous studies [1]. On follow-up visits at 2 and 4 weeks after the initial enrollment visit, 21 other isolations were made. In 10 cases the same serotype was isolated on two or more visits; 5 of these 10 patients had *C. jejuni* serotype 9.

Of the patients who initially did not have *Campylobacter* isolated and for whom follow-up testing was done, stool cultures were positive for *Campylobacter* in 18 (8.3%) of 216 on the 2-week visit and 8 (10.4%) of 77 on the 4-week visit. The serotypes of these isolates were similar in spectrum to the serotypes from the patients who were *Campylobacter*-positive at the initial visit. In addition, 4 and 9 patients initially found to be infected with *Campylobacter* had a new serotype at the 2- and 4-week follow-up visits, respectively. Of the 24 patients from whom *Campylobacter* was isolated on the follow-up visits, 11 (45.8%) were asymptomatic.

*Quantitative microbiology.* The 54 *Campylobacter*-infected children for whom quantitative studies were done shed from $10^1$ to $10^8$ cfu/g of stool (median, $10^6$ cfu/g). We then assessed the relationship between quantitation of *Campylobacter* infections and features of infection (table 1). Twenty-two (40.7%) of the 54 children shed $<10^5$ cfu/g of stool. Children infected with *C. jejuni* had higher fecal excretion than those with other *Campylobacter* species ($P < .001$). Conversely, fecal *Campylobacter* loads were inversely related to the presence of fecal leukocytes; however, this trend did

**Table 1.** Epidemiologic and clinical characteristics of 54 *Campylobacter*-infected patients in relation to quantitative bacteriologic findings.

| Characteristic | $\log_{10}$ *Campylobacter* cfu/g of stool | | | |
|---|---|---|---|---|
| | ≤2.9 ($n$ = 12) | 3.0–4.9 ($n$ = 10) | 5.0–6.9 ($n$ = 22) | 7.0–8.9 ($n$ = 10) |
| Mean age (months) | 19.2 | 14.1 | 13.5 | 11.7* |
| Mixed infections | 25 | 70 | 32 | 20 |
| *Campylobacter jejuni* involved | 25 | 80 | 82 | 100† |
| Fecal WBC | 75 | 70 | 68 | 60 |
| Fecal RBC | 58 | 50 | 59 | 50 |
| Diarrhea for <24 h | 42 | 30 | 50 | 20 |
| Mean no. of stools/24 h | 6.5 | 6.9 | 5.9 | 6.7 |

NOTE. Unless indicated, data are % with characteristic.
* $P$ = .03, $\chi^2$ for trend.
† $P < .001$, $\chi^2$ for trend.

not reach statistical significance ($P = .08$). There was a significant inverse relationship between children's age and the $\log_{10}$ number of *Campylobacter* colony-forming units in stools. Children <2 years of age were significantly ($P = .04$, Fisher's exact test, one-tailed) more likely to excrete higher numbers ($>10^5$) of *Campylobacter* organisms in stools than were older children.

*Serologic results.* Baseline and follow-up sera were available from 56 of 61 *Campylobacter*-infected patients and 31 randomly selected *Shigella*-infected patients (as controls); these were assayed for antibodies against *Campylobacter* group antigens. For each immunoglobulin class, seroconversion occurred significantly ($P < .05$) more often in the *Campylobacter*-infected than in the *Shigella*-infected group.

The *Campylobacter*-infected patients were stratified by age to determine differences in immune response (figure 1). Initial (visit 0) antibody levels were lowest in the children <6 months old, and there was little response during the follow-up period. In those 6–11 months old, the serologic response was substantially greater but rapidly declined in both IgA and IgM; IgG remained elevated at the 4-week follow-up visit. There was a significant increase in visit 0 level for each immunoglobulin class concomitant with increasing age; $P = .003$ (IgG), $P < .001$ (IgM), and $P = .02$ (IgA), analysis of variance for trend (figure 1).

We examined optical density values in the IgA, IgG, and IgM assays for the 61 *Campylobacter*-infected patients in relation to their initial symptoms, using sera from the initial and 2- and 4-week follow-up visits. No significant differences in serologic responses were seen between groups, including those with and without bloody diarrhea, with and without mucus in stools, or with and without fever, nor were differences in responses seen in relation to the level of dehydration at presentation (data not shown). The immune response in IgA, IgG, and IgM did not vary according to severity of illness. However, these analyses were confounded by mixed infections.

On initial presentation, 10 of 61 *Campylobacter*-infected patients had one or more other pathogens present on stool examination; 9 presented with one other pathogen and 1 presented with two other pathogens. When we compared these 10 patients to age-matched patients from whom only *Campylobacter* species were isolated, there was no significant difference in the immune response to *Campylobacter* antigens in any immunoglobulin class.

## Discussion

We isolated *Campylobacter* species from 15% of children with diarrhea, confirming the frequency of these pathogens in Thailand [2, 14]. Our data indicate that the diarrheal illness associated with *Campylobacter* species is expressed as a broad spectrum of symptoms, yet most children have loose stools with mucus, along with fever and vomiting. However, *Campylobacter* infection was clinically indistinguishable from diarrheal illnesses caused by other pathogens that are common in the same population. While bloody diarrhea is seen in more than half of the *Campylobacter*-infected persons who seek medical attention in the United States [15], it was seen in one-quarter of patients in Thailand, predominantly in children <2 years old.

Using extensive bacteriologic protocols to test every stool specimen submitted, we found that 38% of ill children had more than one enteric pathogen isolated, confirming that mixed *Campylobacter* infections are common in the developing world [3, 7]. Age, sex, and symptoms were similar in patients from whom *Campylobacter* organisms were the single pathogen identified and in those with mixed infection, and the prevalence of fecal blood and leukocytes was similar in both groups. Such findings suggest that the *Campylobacter*

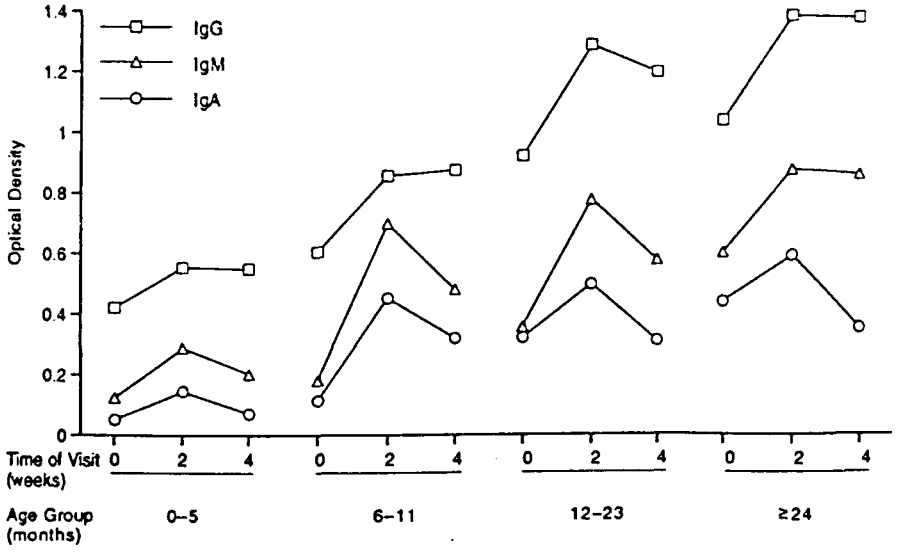


**Figure 1.** *Campylobacter*-specific immune response in children with *Campylobacter* species isolated in setting of acute diarrheal illness, Bangkok. Time of visit refers to initial (O) or 2- or 4-week follow-up visits. Age-related increase in baseline immunoglobulin levels was significant; $P = .003$ (IgG), $P < .001$ (IgM), and $P = .02$ (IgA), analysis of variance for trend. Increase from baseline to 2 weeks was significant ($P < .05$, Wilcoxon signed rank test) for IgG, IgM, and IgA in those 6–11 months, IgA in those 0–5 months, and IgG in those 12–23 months.

species play a role in the illness among persons with mixed infections.

In developed countries, acutely symptomatic *Campylobacter*-infected persons shed $10^6$–$10^9$ cfu/g of stool [16]. An earlier study suggested that the intestinal load of these pathogens may be lower in Thai children and that there is an age-related gradient, as indicated by differential positivity rates with enrichment techniques [1]. In this study, we found that older children excrete fewer *Campylobacter* organisms and less often have grossly bloody stools, findings consistent with the development of immunity. The frequent acquisition of new infections in the population studied demonstrates the hyperendemicity of *Campylobacter* species, providing a basis for the development of immunity.

Because there is not a simple animal model for *Campylobacter* infection, studies in humans are the best approach to understanding the manifestations of infection and immune response. Previous reports from developing countries [1–3] indicate that *Campylobacter* species mainly cause disease in the youngest age groups, with age-related infection rates decreasing with coincident increases in immune response to *Campylobacter* antigens. Studies in volunteers [17] also indicate that *Campylobacter*-specific antibody production is stimulated by infection. Collection of sera from patients who had diarrheal disease in a community in which *Campylobacter* infection rates are high gave us the opportunity to analyze the development of naturally acquired immunity by a variety of parameters.

*Campylobacter* infections occurring in the first 6 months of life induced minimal serologic response. Poor seroresponse may occur because it is a primary response to *Campylobacter* organisms or because of maternal antibodies from placental transfer or from breast feeding. Previous studies in the same population have shown that 38% of children <1 year old received breast milk.

We found that development of *Campylobacter*-specific serum antibodies was age-dependent; older children were more seroresponsive and with maturation, the baseline antibody levels also increased (figure 1). The inverse correlation between rising serum antibody levels and falling rates of infection suggest that previous infection, as measured by presence of serum antibody, protects against subsequent infection with *Campylobacter* species. The age-dependent increase in baseline antibodies also was noted in patients diagnosed with *Shigella* infections (data not shown), indicating that the acquisition of serum antibodies recognizing *Campylobacter* antigens is a general phenomenon in this population, reflecting a high frequency of exposure to *Campylobacter* antigens, regardless of the cause of any particular acute illness.

*Campylobacter* infections occurring after 6 months of age induce the highest serologic responses, stimulated by presumed repeated exposure to the antigens, associated with a secondary or anamnestic-type response [1, 3]. Antibody levels decreased rapidly after their peak and were only slightly above baseline 1 month after infection (figure 1). However, we speculate that the age-related increase in antibody levels results from the cumulative effect of multiple exposures and the parallel development of immunity [3]. This phenomenon occurred in all three antibody classes, so we cannot determine which is the most significant. The age-related decrease in fecal excretion of *Campylobacter* organisms that we observed parallels this development of immunity, as predicted from our earlier study [1]. That the isolation of multiple pathogens did not alter the serologic response to the concomitant *Campylobacter* infection likely reflects ability of the immune system to develop many antigen-specific responses simultaneously.

In conclusion, in a Thai population in which enteric infections are frequent, *Campylobacter* infections are hyperendemic. As children age, their *Campylobacter* infections become milder, they excrete fewer organisms, and *Campylobacter*-specific serum antibodies rise progressively. All of these findings are consistent with the development of immunity, which suggests that development of a vaccine against *Campylobacter* infections should be possible.

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# Capsular Types of *Vibrio vulnificus:* An Analysis of Strains from Clinical and Environmental Sources

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*Vibrio vulnificus* produces a capsular polysaccharide (CPS) that is essential for virulence. CPS from *V. vulnificus* clinical strain MO6-24 has been purified and the structure determined. In preliminary screening with antisera raised to MO6-24 CPS, 4 (19%) of 21 clinical isolates (including MO6-24), but none of 67 environmental *V. vulnificus* isolates, agglutinated with anti-MO6-24 antisera ($P = .003$). CPS was isolated from a subset of 12 clinical and 7 environmental isolates and analyzed by high-performance anion-exchange chromatography and one-dimensional nuclear magnetic resonance. MO6-24 and 1 other serologically positive strain had identical CPS structures; the other 2 serologically positive strains had substitutions in two of four sugar residues. Thirteen other capsular types were identified among the remaining 15 strains from which CPS was extracted.

*Vibrio vulnificus* is a gram-negative bacterium that is ubiquitous in the estuarine environment. It can cause a syndrome of primary septicemia in susceptible hosts, including persons with a history of cirrhosis, alcohol abuse, hemochromatosis, or immunosuppression. Primary septicemia occurs most often after ingestion of raw oysters or other shellfish. After incubation for 24–48 h, patients present with fulminant septicemia, without an apparent focus of infection, that is rapidly and frequently fatal (mortality >50%) [1, 2]. Wound infections associated with exposure to seawater can also occur. These infections may mimic gas gangrene, with metastatic cellulitic lesions progressing to bullae formation, skin necrosis, necrotizing fasciitis, and myositis [2]; sepsis is a not infrequent complication.

Many virulence factors have been suggested for *V. vulnificus*, including production of a polysaccharide capsule [3, 4], expression of extracellular products, such as cytolysin, protease/elastase, and collagenase, and the ability of the organism to acquire iron from the host [5]. Deletion or mutation of genes involved in expression of either the cytolysin or the protease/elastase have resulted in little or no change in the virulence of the organism, as measured by $LD_{50}$ in mice [6, 7]. In contrast, mutants that lack the capsular polysaccharide (CPS) show an increase in $LD_{50}$ of more than four orders of magnitude [4]. This capsule has been shown by our laboratory and others to be important in defense against complement-mediated killing and phagocytosis [3, 4]. Antibodies to the CPS of the infecting organism are found in the serum of some patients who have had *V. vulnificus* septicemia [8].

While polysaccharide capsules serve as a general defense mechanism for bacteria, the virulence and disease manifestations associated with a specific bacterial strain are often dependent on the capsular type or structure (e.g., *Haemophilis*

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