Yersinia enterocolitica Isolated from Two Cohorts of Young Children in Santiago, Chile: Incidence of and Lack of Correlation between Illness and Proposed Virulence Factors

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Yersinia enterocolitica was isolated from children in two cohorts in Santiago, Chile. In a cohort containing a cross section of children aged 0 to 4 years, *Y. enterocolitica* was isolated from stool samples of 1.1% of children with diarrhea and 0.2% of age-matched control children. In a subgroup of this cohort from which weekly stool samples were obtained from all children irrespective of clinical status, 6% of children had asymptomatic *Yersinia* infections. In a birth cohort (with a greater representation of children <1 year of age and a significantly higher rate of diarrhea), *Y. enterocolitica* was isolated from 1.9% of children with diarrhea and 0.6% of controls ($P = 0.05$). Biogroup 1A strains (which lacked traditional phenotypic and molecular markers for pathogenicity) were isolated from seven children with diarrhea but from no control children in the birth cohort ($P = 0.02$). All other isolates, including all isolates from asymptomatic children, were "pathogenic" strains in biogroup 4, serogroup O3; no association between these isolates and occurrence of disease was found. *Y. enterocolitica* is found among young children in Santiago, with asymptomatic infections not uncommon occurrences. However, questions about the association between previously described virulence factors and diarrheal illness remain.

Yersinia enterocolitica is now recognized as an important cause of human disease (1, 3, 4, 8, 16, 28). The organism has been isolated most frequently in temperate areas of the world, with the majority of cases reported from northern Europe and North America (1, 8). While diarrhea is the most commonly recognized clinical manifestation, *Y. enterocolitica* can also cause invasive disease (mesenteric adenitis, septicemia) and has been associated with a variety of autoimmune manifestations, including arthritis and erythema nodosum (1, 8). Only a limited number of biogroups and serogroups are thought to be pathogenic (7). Characteristics shared by these strains include carriage of the *Yersinia* virulence plasmid (conferring calcium dependence and the ability to bind Congo red [7, 23-25]); a negative pyrazinamidase reaction (13); the presence of chromosomal genes for the *Yersinia* heat-stable enterotoxin (*Yersinia* ST) (9); and an invasive phenotype, as indicated by invasion of HEp-2 tissue culture cells and the presence of the *ail* and *inv* chromosomal loci (18, 19).

Despite the widespread recognition of *Y. enterocolitica* as a pathogen, prospective community-based incidence data for this organism are extremely limited (8). Fundamental questions about the clinical significance of many *Y. enterocolitica* isolates also remain; there have now been several studies in which it has not been possible to show a clear correlation between established virulence markers and the occurrence of diarrheal illness (2, 14, 21). In an effort to address these issues, we undertook a study of the epidemiology and pathogenicity of *Y. enterocolitica* among children in two prospectively monitored cohorts in Santiago, Chile.

MATERIALS AND METHODS

(i) **Study design.** Studies were conducted in Santa Julia, a poor, working-class neighborhood in Area Oriente, Santiago, Chile. The neighborhood has a population of 130,000 within an area of 12.3 km². The Chilean National Health Service provides health care to approximately 96% of the population of Santa Julia through a neighborhood health center, Consultorio Santa Julia. By using records maintained at the health center, two cohorts of children were selected for study (10). An initial cohort was selected to provide a cross section of children <48 months of age, with approximately equal numbers of children <12, 12 to 23, 24 to 35, and 36 to 47 months of age. This cross-sectional cohort was monitored for 1 year beginning in January 1987. Three hundred fifty-nine children were in the cohort at the beginning of the study. Because of out-migration, the cohort numbered 330 after 1 year; this represents a total of 4,088 child-months of observation. A second birth cohort was formed beginning in March 1987: approximately 12 infants were enrolled in the cohort each month for 12 months and then monitored for up to 32 months (through October 1989). In this second cohort, there were a total of 3,160 child-months of observation. Because of the way in which the cohorts were structured, the child-months of observation were markedly shifted toward younger children in the birth cohort (Fig. 1).

The household of each child in each cohort was visited twice weekly by a specially trained public health nurse who asked questions about occurrence of diarrhea. Diarrhea was defined as an overt change in the normal stool pattern of the child as characterized by an increase in the frequency (to at least three stools per day) and a decrease in the consistency of stools to an unformed state as noted by the child's

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FIG. 1. *Y. enterocolitica* cohort studies: child-months of observation, by age. Symbols: ■, birth cohort; ▨, cross-sectional cohort.

caretaker. If a child had diarrhea, a stool specimen or rectal swab was obtained on two consecutive days from the child with diarrhea and from a predetermined, age-matched asymptomatic control child in the cohort (two samples per child per episode). Within the cross-sectional cohort, a subgroup of 120 children was randomly selected. In addition to the surveillance described above, these children had a stool specimen collected once each week for culture, irrespective of clinical status.

(ii) **Laboratory methods.** Stool samples were transported in glycerol-phosphate-buffered saline. For the cross-sectional cohort, samples were plated on MacConkey, xylose lysine desoxycholate, and *Salmonella-Shigella* agars, and plates were incubated at 35 to 37°C for 18 to 24 h. Colonies with a morphology suggestive of that of *Yersinia* spp. were picked and speciated on the basis of standard biochemical reactions; the only other potential pathogen identified in these samples was shigellae (10). A more comprehensive evaluation of samples from children in the birth cohort was conducted. In addition to the above media, samples were plated on cefsulodin irgasan novobiocin selective media and incubated at 25°C for 24 to 48 h. For these samples, efforts to identify all other possible bacterial enteric pathogens (including enterotoxigenic, enterohemorrhagic, enteroinvasive, and enteropathogenic *Escherichia coli*), pathogenic protozoa, and rotavirus were also made by using previously described methods (15). Data for isolation rates for shigellae have been reported separately (10).

All *Y. enterocolitica* strains were bityped (7) in Santiago, and serotypes were determined by slide agglutination with polyvalent and monovalent antisera (PROBAC, São Paulo, Brazil). In subsequent testing at the University of Maryland and the University of Melbourne, strains were screened for pyrazinamidase activity (13) and speciation and biotype were confirmed. Serotype designations were confirmed with antisera raised at the University of Melbourne to *Y. enterocolitica* type strains kindly provided by G. Wauters, Université Catholique de Louvain.

All isolates were screened by colony blot hybridization for the *Yersinia* ST gene by utilizing a 22-base synthetic oligo-

nucleotide probe derived in our laboratory from the published nucleotide sequence of the gene (nucleotides 243 to 264, from the sequence as published by Delor et al. [9]). The presence of the *Y. enterocolitica* virulence plasmid was confirmed by hybridization with the no. 6 and 7 *Bam*HI plasmid fragments from *Y. enterocolitica* A2635 (25) and by demonstration of calcium dependence (14, 25). Because of concern that negative results might reflect loss of the virulence plasmid during transport and handling, probe-negative strains were plated onto Congo red agar (23) and Congo red-positive colonies were then rescreened for homology with the plasmid probes and for calcium dependence.

Strains in biogroup 1A were further evaluated for the presence of the *inv* and *ail* chromosomal genes associated with cell invasion (18, 19, 25). Since the *inv* gene may be nonfunctional in nonpathogenic strains (22), strains were also screened for HEp-2 invasiveness (19). The ability of strains to kill mice was tested by inoculating mice (pretreated with iron dextran and desferrioxamine B) intraperitoneally with 4×10^7 bacteria (25). Enterotoxin activity was evaluated by assessing fluid accumulation activity in infant mice (26).

RESULTS

Epidemiologic evaluation. (i) Cross-sectional cohort. In the cross-sectional cohort, 471 episodes of diarrhea were identified during the period of observation, for a rate of 11.5 episodes per 100 child-months of observation. *Y. enterocolitica* was isolated from samples from 5 (1.1%) of the 441 episodes during which cultures were collected (0.12 episodes of *Y. enterocolitica*-associated diarrhea per 100 child-months of observation). The mean age of symptomatic children was 27 months (range, 14 to 39 months). Clinical presentation is summarized in Table 1. Of 441 age-matched controls, 1 child (0.2%) was culture positive for *Y. enterocolitica*; this rate did not differ significantly from that among symptomatic children ($P = 0.22$, McNemar matched-pair analysis).

In the subgroup from which stool samples were routinely obtained on a weekly basis, 18 (0.31%) of 5,723 stool

TABLE 1. Clinical and epidemiologic results, by cohort

Results	Cross-sectional cohort	Birth cohort
No. of episodes of diarrhea	471	702
Rate/100 child-months	11.5	22.2
No. of diarrheal episodes from which stool sample was obtained	441	702
No. of diarrheal stool cultures that were positive for <i>Y. enterocolitica</i> (%)	5 (1.1)	13 (1.9)
Rate/100 child-months	0.12	0.41
No. of stool cultures from age-matched asymptomatic controls that were positive for <i>Y. enterocolitica</i> (%)	1 (0.2)	4 (0.6)
Symptomatic children with <i>Y. enterocolitica</i>		
Mean age, in months (range)	27 (14–39)	13.6 (8–23)
No. of children with diarrhea	5	11 ^a
No. of children with fever $\geq 38^{\circ}\text{C}$	2	1
No. of children with bloody diarrhea	0	0
Mean duration of diarrhea, in days (range)	8.4 (4–10)	11.6 (3–29)

^a The 13 episodes of diarrhea in the infant cohort involved a total of 11 children.

cultures were positive for *Y. enterocolitica*. These 18 positive cultures were from seven asymptomatic children (6% of total children in the subcohort): one child had 5 positive cultures during a 14-week period, one had 3 positive cultures during a 7-week period, and a third had 6 positive cultures during a 10-week period. Counting temporally related positive cultures as a single infection, there were 0.51 asymptomatic *Y. enterocolitica* infections per 100 child-months of observation (rate ratio of incidence of asymptomatic to symptomatic infection = 4.25). The mean age of asymptomatic culture-positive children in the subgroup was 40 months (range, 14 to 57 months).

(ii) **Birth cohort.** In the birth cohort, there were 702

episodes of diarrhea identified during the period of observation (22.2 diarrheal episodes per 100 child-months of observation); this is a significantly higher rate than that found for the older cross-sectional cohort ($P < 0.001$, chi square). *Y. enterocolitica* was isolated from 13 (1.9%) of the 702 diarrheal samples (0.41 *Y. enterocolitica*-associated diarrheal episodes per 100 child-months of observation). The 13 episodes occurred in 11 children, with 1 child having 3 *Y. enterocolitica*-associated diarrheal episodes within a 6-week period. In three instances, other potential pathogens were also identified: *Campylobacter jejuni* was isolated from one stool sample, and giardia were seen in two other samples. The mean age of symptomatic, culture-positive children was 13.6 months (range, 8 to 23 months).

Y. enterocolitica was isolated from stool samples obtained from 4 (0.6%) of the 693 age-matched control child episodes. These four isolates were from three children; one culture-positive, asymptomatic child was selected as a control twice within a 2-month period. When calculated on the basis of episodes, there was an association between isolation of *Y. enterocolitica* and the occurrence of diarrhea ($P = 0.05$, McNemar matched-pair analysis). The mean age of asymptomatic, culture-positive control children was 16.7 months (range, 12 to 25 months).

Laboratory characterization of strains. Thirty isolates were available for further study: 5 case and 1 control isolate from the cross-sectional study, 7 isolates from the subgroup from whom samples were taken on a weekly basis (the initial isolate from each of the seven asymptomatic, culture-positive children), and 13 case and 4 control isolates from the birth cohort.

Seven isolates from children with diarrhea in the birth cohort were pyrazinamidase- and esculin-positive strains of biogroup 1 (designated as biogroup 1A) (Table 2). Isolation of these strains was significantly associated with diarrheal disease ($P = 0.02$, McNemar matched-pair analysis); statistical significance was retained even after exclusion of the child from whom *C. jejuni* was isolated ($P = 0.04$, McNemar matched-pair analysis). There were no obvious differences in the clinical syndromes seen among the children infected with the biogroup 1A strains and among those infected with strains of biogroup 4. None of the biogroup 1A strains hybridized with the AilC or *Yersinia* ST gene probes, all were positive with the Inv probe (but did not invade HEp-2

TABLE 2. Characterization of *Y. enterocolitica* strains of biogroup 1A

Strain no.	Laboratory no.	Serogroup	Binding to Congo red	Hybridization with probe for:					Mouse lethal	HEp-2 invasion ^b	Fluid accumulation ratio ^c	Other possible pathogens present
				Inv(Ye)	AilC	B6 ^a	B7 ^a	ST				
1	937	6	—	+	—	—	—	—	—	0.01	0.144	<i>C. jejuni</i>
2	1001	7, 8	—	+	—	—	—	—	—		0.077	
3	1081	7, 8	—	+	—	—	—	—	—	0.01	0.090	Giardia
4	1109	7,	—	+	—	—	—	—	—	<0.01	0.076	
5	1114	10	—	+	—	—	—	—	—	0.08	0.057	
6	1267	7, 8	—	+	—	—	—	—	—	0.14	0.062	
7	1350	10	—	+	—	—	—	—	—	0.01	0.064	
Controls	A2635	8	+	+	+	+	+	+	+	NT ^d		
	A2635c ^e	8	—	+	+	—	—	+	—	20.0		

^a B6, *Bam*HI no. 6 fragment from virulence plasmid of *Y. enterocolitica* A2635; B7, *Bam*HI no. 7 fragment.

^b Percent of the bacterial inoculum recovered from cells exposed to gentamicin (using the method of Miller et al. [19]).

^c Mean ratio of gut weight to remaining body weight in the infant mouse assay; ratio ≥ 0.09 = positive; ratio < 0.065 = negative.

^d NT, not tested.

^e Plasmid-cured derivative of A2635 (i.e., strain lacking the *Y. enterocolitica* virulence plasmid).

cells), and none were mouse lethal. However, two strains (1 and 3) gave a positive response in the infant mouse assay for enterotoxin, with two additional strains (2 and 4) giving intermediate results.

All other isolates (including all isolates from asymptomatic children in both cohorts) were biogroup 4, serotype O3 and were pyrazinamidase negative. With the exception of two case and one control isolate, all carried the *Y. enterocolitica* virulence plasmid, on the basis of hybridization with our genetic probes and calcium dependence. All strains hybridized with the synthetic oligonucleotide probe for *Yersinia* ST. When the analysis was restricted to these isolates, no association with occurrence of diarrhea was seen.

DISCUSSION

The unique feature of this study was the availability of prospective community-based data for rates of isolation of *Y. enterocolitica* from both symptomatic and asymptomatic children. Earlier studies have identified cases through clinic- or laboratory-based surveillance, making direct comparisons of data difficult; comparisons are also complicated by differences in the age structures of the populations sampled. However, within these limitations, rates of isolation of *Y. enterocolitica* from young children with diarrhea in Santiago appear to be comparable to those seen in other temperate areas. In an outpatient study conducted in Baltimore, Md., *Y. enterocolitica* was isolated from 2% of children less than 2 years of age with diarrhea (15). Similar isolation rates from children have been reported in hospital laboratory-based studies in Montreal, Canada (2.8% of all diarrheal stool cultures positive for *Y. enterocolitica* [16]; median age of culture-positive children, 24 months), and Siena, Italy (1.4% of stool cultures among children ≤ 14 years of age positive for *Y. enterocolitica* [20]). In contrast, in a study in Bangladesh, the isolation rate among children less than 7 years of age with diarrhea was 0.06% (6).

A more striking finding was the isolation of *Y. enterocolitica* from 6% of a subgroup of 120 children from whom stool cultures were obtained on a weekly basis. Despite intermittent carriage of *Y. enterocolitica* for periods of up to 14 weeks, none of these children were symptomatic. We did not look specifically at possible sources of infection; however, prolonged excretion of *Y. enterocolitica* by asymptomatic young children would provide an appropriate setting for person-to-person spread within families, possibly through an intervening food vehicle (8, 12, 17). The reasons for the high rate of asymptomatic carriage are not completely clear. If children were acquiring resistance to *Y. enterocolitica* (or, at least, to biogroup 4, serogroup O3 strains) by virtue of prior exposure to the organism, one might expect that children with symptomatic disease would be younger than children who were asymptomatic carriers. By using *C. jejuni* as an example, it has been shown that in developing countries the ratio of *C. jejuni* illness to infection is often inversely proportional to age (5, 11, 27). While our study design and sample size make statistical comparisons difficult, the mean age of asymptomatic, infected children in this study was higher than that of symptomatic children from whom *Y. enterocolitica* was isolated.

Asymptomatic infection with *Y. enterocolitica* in children in Santiago was not attributable to infection with a nonpathogenic strain. *Y. enterocolitica* isolates from asymptomatic children were all from a pathogenic biogroup and serogroup, and, with few exceptions, had all of the characteristics associated with pathogenic strains (7, 9, 13, 14, 18,

19, 21, 23–25). In contrast, a group of biogroup 1A strains, which should not have been pathogenic on the basis of traditional criteria, were significantly associated with diarrhea. At least two of these isolates (neither of which hybridized with the *Yersinia* ST gene probe) were enterotoxic in infant mice, suggesting that these strains have mechanisms, independent of cell invasion, by which they may be able to cause illness. In the absence of human volunteer studies, we cannot say with certainty that biogroup 1A strains did or could cause diarrhea. However, there have now been several epidemiologic studies that have raised questions about the link between traditional criteria for pathogenicity and diarrheal disease. Nobel et al. (21), reporting from Vancouver, British Columbia, found that the majority of *Y. enterocolitica* isolates from patients with diarrhea at their hospital did not carry standard virulence markers. Bissett et al. (2), at the California Department of Health Services, reported that over 40% of *Yersinia* isolates submitted to the state health department laboratory belonged to serogroups generally considered nonpathogenic; many of these isolates were from persons with mild to moderate gastroenteritis. Our study raises similar questions and suggests that our understanding of the relationship between "pathogenic" *Y. enterocolitica* and diarrheal disease is still incomplete.

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