Prevalence of Antibodies to Norwalk Virus in England: Detection by Enzyme-Linked Immunosorbent Assay Using Baculovirus-Expressed Norwalk Virus Capsid Antigen

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A total of 3,250 serum specimens collected in England in 1991 and 1992 were tested by an indirect enzyme-linked immunosorbent assay for antibody to Norwalk virus using baculovirus-expressed capsid antigen, and 2,382 (73.3%) were positive. The prevalence of Norwalk virus antibody differed regionally. It was lowest (24.6%) in 6- to 11-month-old infants and increased to 89.7% in persons over 60 years old.

Norwalk virus, although associated with winter vomiting disease (1), is a major cause of food-borne epidemics of nonbacterial gastroenteritis in the United Kingdom and worldwide (15). It has been shown to be the etiologic agent of gastroenteritis in more than 90% of food-borne outbreaks in which a virus has been identified (21). Norwalk-like viruses such as Snow Mountain agent (6), Hawaii agent (17), and Taunton agent (4) all produce Norwalk-like illness and are biologically and morphologically similar to Norwalk virus (15). The true incidence of infections with Norwalk and Norwalk-like viruses is unknown, as the illness is generally mild, and therefore many cases of viral gastroenteritis go unreported (15).

Although various studies have been carried out to investigate the prevalence of antibody to Norwalk virus (2, 3, 5, 7, 10, 16, 22, 23) or the incidence of Norwalk virus infection (11, 22), the inability to grow the virus *in vitro* has meant that an abundant and consistent supply of Norwalk virus antigen has so far not been available. Therefore, epidemiological studies on a large scale are lacking.

The recent cloning and characterization of Norwalk virus-specific cDNA (13, 19) have led to the production of immunologically reactive recombinant Norwalk virus antigen particles (rNV) (12, 14). In the laboratory of one of us (M. K. Estes), a clone comprising the total sequence for the capsid has been expressed in baculovirus-infected insect cells, and empty capsids were formed from this expressed protein (14). The antigenicity of this recombinant antigen was comparable to that of the native antigen, and it was shown to react sensitively and specifically with well-characterized sera obtained from previous Norwalk virus-associated outbreaks (9). In the present study, this Norwalk virus capsid antigen was used in an indirect enzyme-linked immunosorbent assay (ELISA) to determine the prevalence of Norwalk virus antibody in several subpopulations in England.

In total, 3,250 serum samples were collected between January 1991 and May 1992 from hospital patients, patients attending outpatient clinics, and healthy blood donors in the northwest (Preston and Manchester), Yorkshire (Leeds), West Midlands (Birmingham), East Anglia (Cambridge), Berkshire (Reading), Kent (Ashford), and Devon (Exeter)

regions of England. All sera were selected at random. With the exception of those from East Anglia, the sera were from the collection of the ongoing Public Health Laboratory Service combined mumps, measles, and rubella vaccine surveillance study (20). There was no connection between serum collection and diarrheic illness. The individuals tested ranged from 1 day to 100 years of age; 49.6% were female, and 50.4% were male.

To perform the Norwalk virus immunoglobulin G ELISA, each well of a 96-well flat-bottomed Falcon microtiter plate (Becton Dickinson UK Ltd., Oxford, United Kingdom) was coated with 100 μ l of baculovirus-expressed Norwalk virus capsid antigen (12, 14) (1 μ g/ml in 0.05 M carbonate-bicarbonate buffer [pH 9.6]) and incubated at room temperature for 4 h. The wells were then washed once with phosphate-buffered saline (PBS), pH 7.2, containing 0.05% Tween 20 and blocked at 4°C overnight with 200 μ l of PBS

TABLE 1. Prevalence by age of Norwalk virus immunoglobulin G antibody^a

Age range	No. of samples tested	No. of samples antibody positive	% Antibody positive ^b
<6 mo	109	82	75.2
6-11 mo	73	18	24.6
12-23 mo	126	61	48.4*
24-35 mo	138	63	45.6
36-47 mo	108	58	53.7
48-59 mo	137	75	54.7
5-9 yr	584	410	70.2*
10-19 yr	710	532	74.9
20-29 yr	495	403	81.4**
30-39 yr	276	236	85.5
40-49 yr	131	120	91.6
50-59 yr	91	80	87.9
60-69 yr	63	53	84.1
70-79 yr	96	90	93.7
80-89 yr	62	56	90.3
≥90 yr	51	45	87.1
Total	3,250	2,382	73.3

^a All results are for serum samples tested at a dilution of 1:100.

^b Underlined values are significantly different (chi-square test; *, $P < 0.001$; **, $P < 0.01$) from those of the previous age group.

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containing 5% skim milk powder. The wells were washed two times as described above, and 100 μ l of patient's serum, diluted 1:100 in PBS containing 1% skim milk powder, was added. Norwalk virus antibody-negative and antibody-positive sera, shown to have optical densities of <0.100 and >1.000 , respectively, were included in each plate. Serum samples were not added to the first two wells, which contained conjugate and substrate controls. The plate then was incubated for 2 h at 37°C before being washed six times as described above. One hundred microliters of horseradish peroxidase-conjugated rabbit anti-human immunoglobulin G (DAKO Ltd., High Wycombe, United Kingdom), diluted 1:1,000 in PBS containing 1% skim milk powder, was added to each well except the substrate control well, and the plate was incubated at 37°C for 2 h. The plate then was washed six times as described above, and 100 μ l of tetramethylbenzidine (0.1 mg/ml in 0.05 M phosphate-citrate buffer [pH 5.0] [Sigma Chemical Company, Poole, United Kingdom]) was

added to each well. After incubation at room temperature for 10 min, when the positive control serum had developed maximum color intensity, the reaction was stopped with 2 M sulfuric acid, and the optical density was read at a 450-nm wavelength. Serum samples with an optical density of ≥ 3 times the mean optical density of the negative control were defined as antibody positive.

The prevalence of Norwalk virus antibody was calculated for each age group (see Table 1), and statistically significant differences between age groups and geographical regions were determined by the chi-square test.

A total of 2,382 of 3,250 (73.3%) serum specimens were positive for Norwalk virus antibody. The prevalence of Norwalk virus antibody by age group is shown in Table 1. Antibody was found in 82 of 109 (75.2%) samples collected from infants less than 6 months old, but only 18 of 73 (24.6%) samples collected from infants between 6 and 11 months old had Norwalk virus antibody. The prevalence of antibody in

FIG. 1. Geographic locations of the laboratories from which samples were collected.

TABLE 2. Regional differences in age-related Norwalk virus immunoglobulin G antibody prevalence

Age range	Results ^a for indicated region (laboratory location)															
	Northwest (Preston)		Northwest (Manchester)		Yorkshire (Leeds)		West Midlands (Birmingham)		East Anglia (Cambridge)		Berkshire (Reading)		Kent (Ashford)		Devon (Exeter)	
	No.	% Pos.	No.	% Pos.	No.	% Pos.	No.	% Pos.	No.	% Pos.	No.	% Pos.	No.	% Pos.	No.	% Pos.
<6 mo	72	76.3	0		0		13	53.8	24	82.3	0		0		0	
6–11 mo	43	30.2	0		0		15	33.3	15	0.0	0		0		0	
12–23 mo	27	59.3	20	60.0	13	38.5	22	40.9	13	46.1	21	52.3	2	50.0	8	12.5
24–35 mo	40	52.5	14	57.1	7	42.8	29	27.6	16	50.0	20	45.0	6	100.0	6	0.0
36–47 mo	21	47.6	8	75.0	17	41.2	25	64.0	10	40.0	15	46.7	9	66.7	3	66.7
48–59 mo	33	54.5	16	56.2	3	66.6	19	68.4	9	44.4	15	46.7	27	51.9	15	53.3
5–9 yr	117	71.8	79	74.6	54	74.1	93	68.8	21	66.7	106	69.8	77	67.5	37	62.1
10–19 yr	178	84.2	129	79.1	45	68.9	157	72.6	20	60.0	59	74.6	79	73.4	43	48.8
20–29 yr	68	79.4	143	77.6	0		126	80.2	70	85.7	0		83	89.2	5	60.0
30–39 yr	35	88.6	68	80.8	0		60	90.0	57	84.2	0		49	85.7	7	85.7
40–49 yr	12	100.0	21	80.9	0		16	81.3	79	94.9	0		3	100.0	0	
50–59 yr	0		32	84.3	0		12	75.0	46	93.4	0		1	100.0	0	
60–69 yr	0		18	72.2	0		13	84.6	28	92.8	0		4	100.0	0	
70–79 yr	0		12	83.3	0		18	88.8	52	96.2	0		14	100.0	0	
80–89 yr	0		1	100.0	0		8	62.5	34	97.0	0		19	89.5	0	
>90 yr	23	86.9	0		0		1	100.0	23	86.9	0		4	100.0	0	
Total	669		561		139		627		517		236		377		124	

^a No., number of samples; % Pos., percent positive.

509 children of 1 to 4 years of age was 50.5% and increased ($P < 0.001$) to 70.2% in 584 5- to 9-year-old children. A further increase ($P < 0.001$) to 81.4% was seen among 495 20- to 29-year-old adults, and the prevalence continued to rise slowly for older adults, with 227 of 253 (89.7%) adults older than 60 years being antibody positive.

A limited number of sera that were collected from the 6-month- to 5-year-old children and were Norwalk virus antibody negative at a 1:100 dilution were retested at 1:20 and 1:50 dilutions. A few of these sera scored weakly positive at the 1:50 dilution (within 10% of the assay cutoff point), but no significant increase of seropositivity in these age groups was recorded when these lower dilutions were tested.

Seroepidemiological studies to determine the age prevalence of Norwalk virus antibody have been performed with serum samples collected in several countries. The majority of these studies have examined the prevalence of Norwalk virus antibodies in young children. In studies performed on serum samples collected from children in Thailand (7), Panama (23), Taiwan (5), the Philippines (5), the United States (10), and Bangladesh (2), the prevalence of Norwalk virus antibodies ranged from 10% at 2 years of age and 60% at 5 years of age in Taiwan to 80% at 2 years of age and 98% at 5 in Panama. In a study performed with serum samples collected from adults in the United States, Belgium, Switzerland, Yugoslavia, Bangladesh, Ecuador, and Nepal (9), the seroprevalence of Norwalk virus antibodies was <50% in the 18- to 19-year-old age group in Yugoslavia and >80% in the same age group in Ecuador and was >60% in those over 50 years of age in all countries studied.

More-detailed analysis of the data of the present study (Table 1) indicated that the high prevalence of Norwalk virus antibody detected in infants less than 6 months old (75.2%) most likely reflected the presence of maternal antibodies. There was a statistically significant increase ($P < 0.001$) in prevalence from 24.6% in 6- to 11-month-old infants to

48.4% in 1- to 2-year-old children. This increase may be associated with children becoming more mobile and having more contact with siblings. The seroprevalence then remained relatively static to the age of 5 years. The next significant increase ($P < 0.001$) in Norwalk virus antibody prevalence occurred at 5 years of age, when 69 of 97 (70.2%) children (part of the 5- to 9-year-old group [Table 1]) had detectable antibody. This coincides with children's starting school. The prevalence increased only slightly throughout the teenage years. A further significant increase ($P < 0.01$) in antibody prevalence was detected in the 20- to 29-year-old group (81.4%) (Table 1); still-susceptible individuals in this age group may acquire primary infections from their recently infected young children. The prevalence remained high in older groups, with 87.1% of the ≥90-year-old population being Norwalk virus antibody positive.

In order to determine whether regional differences in the prevalence of Norwalk virus antibody existed, the results were analyzed according to the geographic area of collection (Fig. 1). Some regional differences in the prevalence of Norwalk virus antibodies were recorded (Table 2). Results obtained from samples collected in laboratories at Preston and Birmingham, whose catchment areas cover the large urban populations in the northwest of England, indicate that infection is acquired earlier in life there than in the more rural areas in the south of England, such as East Anglia, served by the Cambridge laboratory. This is demonstrated in the results for the 6- to 11-month-old infants; 30.2 and 33.3% of the sera from children in this age group from Preston and Birmingham, respectively, were Norwalk virus antibody positive, whereas none of the sera collected from 6- to 11-month-old infants in Cambridge was positive ($P < 0.001$). In the 12- to 23-month-old children, by contrast, there were no significant ($P < 0.05$) differences in antibody prevalence between urban regions (Preston, Manchester, Leeds, and Birmingham) and rural areas (Cambridge, Reading, Ashford, and Exeter) (Table 2). Although the rate at which Norwalk

virus infection is acquired varies across regions, more than 80% of the population between the ages of 30 and 40 years in all of the regions studied had Norwalk virus antibodies. Although the high prevalence of >80% detected in the population ≥ 90 years of age may be due to the sensitivity of the assay, it also may reflect reinfection in an elderly population with waning antibody levels, as has been demonstrated with rotavirus (8).

Individuals are at risk of infection with Norwalk virus from the time when maternal antibodies wane. Although infection with Norwalk virus can occur at any time after this, significant rises in the prevalence of Norwalk virus antibody may correspond with specific events. At around 1 year of age, when a child is becoming mobile; at 3 to 5 years of age, when a child may attend a play group or nursery school; at 5 years of age, when a child starts attending school; at 11 or 12 years of age, when a child changes schools; and between 20 and 30 years of age, when changes in life-style or contact with one's own children may be the source of infection, Norwalk virus antibody prevalence has shown significant increases.

Differences in the reported age prevalence of Norwalk virus antibodies in this and other studies (2, 3) may be caused by differences in assay sensitivity (16) or the choice of antigen or geographic location (10). It was recently shown that the baculovirus-expressed rNV and native Norwalk virus reacted very similarly in immune electron microscopy tests and ELISAs (9), and baculovirus-expressed rNV now provides an ample quantity of antigen for large epidemiological studies. Norwalk virus is one of a group of Norwalk-like agents which includes Snow Mountain agent, Hawaii agent, Taunton agent, and other related viruses. Although antigenic relatedness among the Norwalk-like agents has been demonstrated, it may be restricted to a one-way cross-reaction (18): in volunteer studies with Norwalk virus and Snow Mountain agent, those challenged with Norwalk virus had rises in titers of heterologous serum antibody to Snow Mountain agent, but this did not occur in the reverse situation. Therefore, high prevalences of Norwalk virus antibodies, as seen in this study, may reflect histories of infection with several different Norwalk-like agents.

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