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Molecular Epidemiology of Norovirus Infections in Stockholm, Sweden, during the Years 2000 to 2003: Association of the GGIIb Genetic Cluster with Infection in Children

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The incidence of norovirus-associated gastroenteritis and the molecular epidemiology of norovirus strains were studied during three seasons (2000-2001, 2001-2002, and 2002-2003) among patients of all ages, mainly from the Stockholm region in Sweden. A total of 3,252 fecal samples were analyzed by reverse transcription-PCR. The incidences of norovirus infection among adults were 23, 26, and 30% during the three seasons studied and 18, 11, and 15% among children 0 to 15 years of age. During the first season, all norovirus strains detected by PCR were typed either by reverse line blot hybridization or nucleotide sequence analysis. During the two successive seasons, a total of 60 norovirus-positive strains from the beginning, peak, and end of the seasons were selected for nucleotide sequence analysis. We identified two dominant norovirus variants over the seasons: a new norovirus variant, recently described as the GGIIb genetic cluster, dominated among children during the first season, and during the following two seasons, a GGII-4 variant dominated. Our data suggest that norovirus infections are common, not only among adults, but also among children, and that some strains may predominantly affect children.

Human norovirus infections have become an increasing problem in health care units and societies around the world. The human noroviruses cause acute gastroenteritis and are considered to be the major cause of epidemic nonbacterial gastroenteritis (10, 13). These viruses have a high attack rate and are stable outside the host, and a low infectious dose is required to cause infection. Norovirus infections have a large economic impact, since wards must be closed for several days in order to stop the virus from spreading in hospitals and nursing home settings.

Human noroviruses belong to the family *Caliciviridae*, which is divided into four genera, *Norovirus* and *Sapovirus*, which cause human infections, and *Lagovirus* and *Vesivirus*, which are associated with veterinary infections (3, 19). The *Norovirus* genus is split into two genogroups, I and II (1, 2, 26, 27). These two major genetic groups each consist of several defined genetic clusters, as illustrated in Table 1 (2, 7, 26). Apart from these, undefined genetic clusters, such as the recently described GGIIb (4, 17), exist. Moreover, a new genogroup has been proposed, GGIV, which contains members of the Alphatron genetic cluster (5). The circulating norovirus strains are classified through comparison to prototype strains belonging to these genetic clusters (Table 1) (2, 7, 26). The most prevalent circulating strains belong to genogroup II (4, 8, 13, 21, 23, 24). During recent seasons, the majority of outbreaks in Europe have been caused by strains belonging to the genogroup II-4 genetic cluster, and since January 2002, a new genetic variant of this cluster has emerged (15).

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In a Swedish study, it has been shown that a significant portion of the acute gastroenteritis cases in older children and adults are caused by norovirus (10). However, the incidence among small children in Sweden remains unknown. The incidence of norovirus infections in young children has been investigated in different regions worldwide. Several reports indicate that 7 to 9% of acute gastroenteritis cases among young children are caused by norovirus (12, 16, 20, 22). However, other studies have reported higher incidence rates, ranging from 15.7 to 20% (6, 11, 18).

In the year 2000, we introduced PCR for diagnosis of norovirus infections in our laboratory. Since its introduction, we have received an increasing number of fecal specimens for diagnosis of norovirus infections. The 2002-2003 season was associated with a very marked increase in the number of norovirus outbreaks all over Sweden, which was reflected in an increased number of specimens sent to the laboratory. The aim of the present study was to follow the incidence of norovirus infections among adults and children in the Stockholm region of Sweden during the years 2000 to 2003 and to identify the predominant strains circulating during that period.

MATERIALS AND METHODS

Specimens. Fecal specimens were received for diagnosis of norovirus infections from a total number of 3,252 patients with gastroenteritis from 2000 to 2003 (Table 2). The patients were mainly from the Stockholm region of Sweden, and in this study, each fecal specimen represents a single patient.

During the 2000-2001 season, all norovirus-positive reverse transcription (RT)-PCR specimens collected (except one) were subjected to reverse line blot hybridization (RLB) for typing (25). However, 47 out of 86 specimens were not completely resolved by RLB typing. Thirty of those specimens were available for nucleotide sequencing of the polymerase gene (34% of all positive specimens).

From each of the two seasons 2001-2002 and 2002-2003, 30 randomly chosen norovirus-positive PCR specimens from the beginning, peak, and end of the

TABLE 1. Norovirus genogroups and genetic clusters[a]

Genogroup	Genetic cluster	Prototype strain
I	1	Norwalk/1968/US
	2	Southampton/1991/UK
	3	Desert Shield 395/1990/SA
	4	Chiba 407/1987/JP
	5	Musgrove/1989/UK
	6	Hesse 3/1997/GE
	7	Winchester/1994/UK
II	1	Hawaii/1971/US
	2	Melksham/1994/UK
	3	Toronto 24/1991/CA
	4	Bristol/1993/UK (Lordsdale/1993/UK)
	5	Hillingdon/1990/UK
	6	Seacroft/1990/UK
	7	Leeds/1990/UK

[a] Grouping according to reference 7.

season were subjected to nucleotide sequencing of the polymerase gene (Table 2). This represents 18 and 5%, respectively, of all PCR-positive specimens during each season. A season was defined as starting in August and ending in July the following year. The positive specimens were selected so that none of the specimens were from patients in the same hospital department or health care unit.

RT-PCR. The specimens were prepared as 20% suspensions in phosphate-buffered saline (pH 7.4). After vortexing and centrifugation at 1,100 to 1,200 × *g*, the supernatant was used in either manual extraction (QIAamp Viral RNA Mini Kit; QIAGEN) or automated extraction using BioRobot M48 (MagAttract Viral RNA M48 kit; QIAGEN). The RNA extracts were immediately tested for norovirus by RT-PCR or stored at −70°C until they were tested. The RT-PCR was performed in one step using the primers previously published by Vinjé and Koopmans (24). The JV12a (5′-ATA CCA CTA TGA TGC AGA TTA) and JV13b (5′-TCA TCA TCA CCA TAG AAA GAG) primers target a 326-bp region of the polymerase gene that is widely used for norovirus typing (24). The JV13b primer was 5′ biotinylated for use in downstream RLB assays. RT-PCR was performed in a final volume of 50 μl containing 4 μl of 10× PCR buffer II (100 mM Tris-HCl, 500 mM KCl, [pH 8.3] [Applied Biosystems]), 1 μl of 5× RT buffer (50 mM Tris-HCl, 250 mM KCl, 20 mM $MgCl_2$, 50 mM dithiothreitol [pH 8.3]), 2 μl of $MgCl_2$ (25 mM), 200 μM deoxynucleoside triphosphate, 0.3 μM (each) primer, 20 U of RNasin (Promega), 5 U of avian myeloblastosis virus reverse transcriptase (Promega), 2 U of AmpliTaq (Perkin-Elmer Applied Biosystems), and 10 μl of extracted RNA. The cycling involved reverse transcription at 42°C for 1 h, followed by denaturation at 94°C for 3 min and then 35 cycles at 94° for 45 s, 42°C for 45 s, and 72°C for 1 min. A final elongation step at 72°C for 10 min was performed. The amplified products were visualized by ethidium bromide staining after agarose gel electrophoresis.

RLB. The biotinylated PCR products were typed by RLB as described by Vinjé and Koopmans (25). Briefly, heat-denatured PCR products were allowed to hybridize to a panel of 18 immobilized oligonucleotides representing genogroup I or II and 15 different genetic clusters. After hybridization and washing, the bound PCR products were incubated with diluted streptavidin-peroxidase conjugate. Finally, the PCR products were detected by an enhanced chemiluminiscence reaction of luminol (Amersham), and the blots were exposed to an X-ray film (Hyperfilm; Amersham).

Nucleotide sequence analysis. The sequencing of PCR products was done with an ABI PRISM BigDye Terminator cycle-sequencing kit, version 3.0, on an automated sequencer (ABI PRISM 3100-*Avant* Genetic Analyzer; Applied Biosystems). Prior to the nucleotide-sequencing reaction, PCR products were purified on spin columns (QIAquick PCR Purification; QIAGEN) according to the manufacturer's instructions. The PCR primers JV12a and JV13b were used as sequencing primers. Nucleotide sequences were imported to the BioEdit Sequence Alignment Editor (9), and multiple alignments were performed using the Clustal W algorithm. The phylogenetic tree was constructed based on 310 nucleotides from the partial RNA polymerase gene using phylogenetic and molecular evolutionary analyses conducted by MEGA version 2.1 (14).

Nucleotide sequence accession numbers. The nucleotide sequence data have been submitted to EMBL and assigned accession numbers AJ626557 to AJ626645.

RESULTS

Incidence of norovirus infections. During the three seasons studied, fecal specimens for norovirus diagnostics were received from a total of 3,252 patients, and 859 (26%) were positive by norovirus RT-PCR (Table 2 and Fig. 1). During the first season, 53% of all specimens were collected from children 0 to 15 years of age. In the two following seasons, the fecal specimens from adults increased dramatically while the number of samples from children remained constant (Fig. 2). The incidence of norovirus infections in the total material increased from 20 to 29% during the three seasons (Table 2), and the peak incidences occurred in March (2001), February (2002), and January (2003) (Fig. 1). The norovirus-positive rate among adults was 23% in the first season, 26% during the second season, and 30% during the third season compared to 18, 11, and 15% among children.

RLB typing. From the 2000-2001 season, 86 out of 87 norovirus-positive PCR products were hybridized to a panel of oligonucleotides targeting two genogroups and 15 genetic clusters (25). As shown in Table 3, 39 out of 86 specimens were successfully typed by the RLB method, while 47 specimens could be typed only to genogroup level (genogroup II). Twenty-nine out of 39 (74%) of the RLB-typed specimens were GGII-4-like. The GGII-positive specimens that could not be further typed were from 36 children and 11 adult patients. Only 5 out of 41 specimens from children matched the RLB panel, and of those, 4 were GGII-4-like and one was GGI-6-like. The last originated from a food-borne outbreak. One positive specimen detected at the end of the season was not subjected to RLB typing.

TABLE 2. Number of patients (adults and children) analyzed for norovirus during three seasons (2000 to 2003) and number of adults and children whose norovirus strains were nucleotide sequenced

Parameter	Value for season[a]:		
	2000-2001	2001-2002	2002-2003
Norovirus-positive patients/total no. of patients	87/429 (20%)	160/690 (23%)	612/2133 (29%)
Norovirus-positive adults/total no. of adults	46/203 (23%)	146/558 (26%)	581/1925 (30%)
Norovirus-positive children <15 years/total no. of children	41/226 (18%)	14/132 (11%)	31/208 (15%)
Total no. of nucleotide-sequenced norovirus-positive specimens	30/87 (34%)	29[b]/160 (18%)	30/612 (5%)
Nucleotide-sequenced adult specimens	6/30 (20%)	24/29 (83%)	27/30 (90%)
Nucleotide-sequenced child specimens	24/30 (80%)	5/29 (17%)	3/30 (10%)

[a] A season was defined as starting in August and ending in July the following year.
[b] One patient specimens failed in nucleotide sequence analysis.

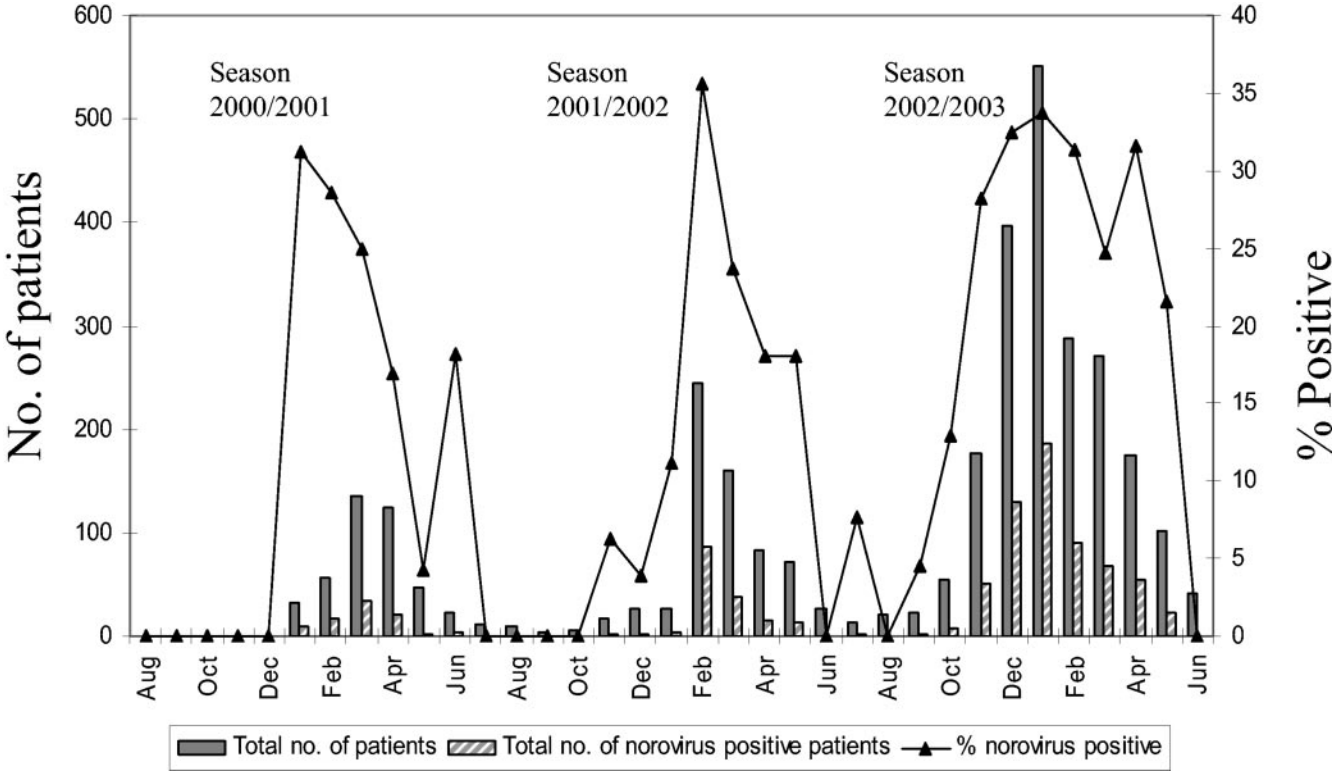

FIG. 1. Prevalences of norovirus infections during three successive seasons (2000 to 2003).

Nucleotide sequence analysis. From the first season, 30 of 47 (64%) specimens in the unresolved genogroup II, which could not be associated with a specific genetic cluster by RLB, were available for nucleotide sequence analysis. We found that these specimens constituted a norovirus cluster, GGIIb, which was also found in Spain (4) and in Stockholm (17) and Gothenburg, Sweden, during the same period (accession numbers AJ487474, AJ487794, AJ487795, and AF365989). These sequences showed 97 to 98% nucleotide sequence similarity to the GGIIb variants but only 84% nucleotide sequence similarity to the Lordsdale strain (GGII-4).

During the second and third seasons, there was a complete dominance of GGII-4 strains, as they were found in 49 of 59 (83%) sequenced strains (Table 4). They showed ~90% nu-

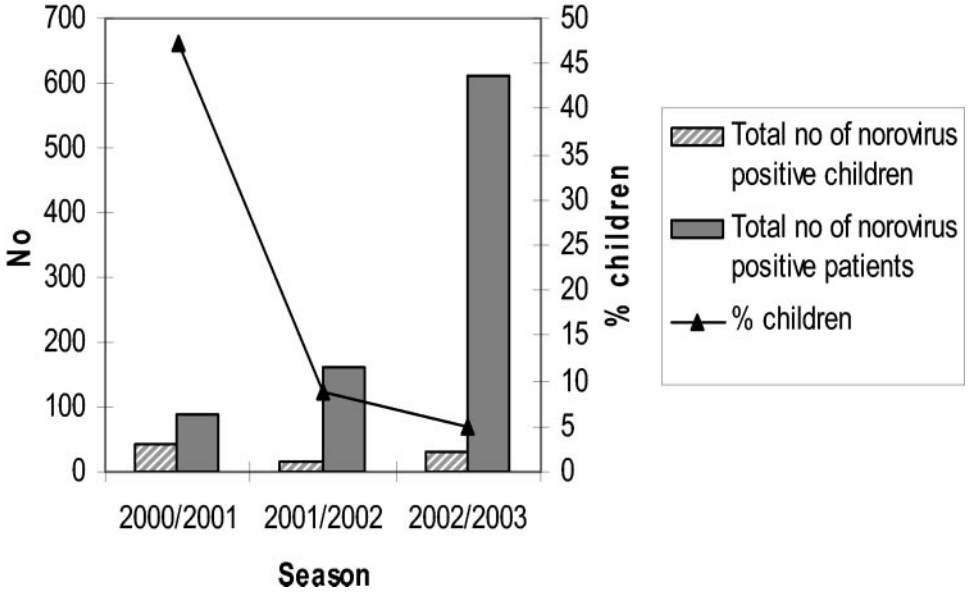

FIG. 2. Number of norovirus-infected children in relation to total number of diagnosed patients.

TABLE 3. Results from RLB hybridization of positive norovirus strains from the 2000-2001 season

Genogroup	Strain	No. positive		
		Adults (n = 46)	Children < 15 yrs (n = 41)	Total (n = 87)
I	Queen Arms (GGI-4)	2		2
	Sindlesham (GGI-6)	1	1	2
II	Not subtyped GGII	11	36	47
	Hawaii (GGII-1)	3		3
	Melksham (GGII-2)	2		2
	Lordsdale (GGII-4)	25	4	29
	Rotterdam[a]	1		1
	Not determined	1		1

[a] Genetic cluster within GGII unknown.

cleotide sequence homology to the Lordsdale strain (GGII-4). The most closely related strain was a norovirus previously characterized in Japan in 1996, with 98 to 99% nucleotide sequence similarity (accession number AB089860). In one recent publication, a specific nucleotide sequence motif (starting position 4820 relative to Norwalk virus M87661) has been described to vary among GGII-4 variants (15). During the whole 2001-2002 season, this motif was AATTTG in the majority of our GGII-4 strains. However, the dominant nucleotide sequence motif in Europe before 2002 was AACTTG (15). After 2002, a nucleotide sequence motif shift to AATCTG was found in strains from all over Europe. The same shift was also recognized in our GGII-4 strains from the beginning of the 2002-2003 season (Table 4).

Apart from GGII-4 strains, the strains from the GGIIb cluster were observed at the end of the 2001-2002 season and at the beginning of the 2002-2003 season. Other findings from these seasons were one strain that turned out to be Fort Lauderdale-like, a proposed GGIV member (5), and one strain closely related to GGI-3 (97% nucleotide homology to accession number AY038598). Moreover, we found strains that were related to GGII-2, one strain related to GGI-2, and one GGI strain (98% nucleotide sequence similarity to accession number AB044359). We failed to retrieve nucleotide sequence data from one of the positive patient specimens.

Based on nucleotide sequence data from the polymerase gene, we constructed a genogroup II phylogenetic tree from aligned nucleotide sequences with Mega2.1 software (Fig. 3). The tree was rooted by the Mexico strain (GGII-3; accession number U22498). During the tree modeling, we tried to break up groups by entering close relatives of the group members (retrieved from nucleotide data banks with the BLAST tool). Finally, the tree was tested by a bootstrap analysis (bootstrap value, 100). We identified several groups. The GGIIb variants from the 2000-2001 season separated into two clusters, although the bootstrap values did not support such a division. All GGII-4 strains formed a separate cluster that branched into one major cluster surrounded by several smaller ones. The GGII-4 strains from 2001-2002 and 2002-2003 grouped into two closely related clusters, although the bootstrap values did not confirm the distinction (Fig. 3).

DISCUSSION

A striking increase in norovirus outbreaks in the 2001-2002 season was recognized all over Europe, and it has been correlated with an emerging new variant, GGII-4 (15). The sudden rise in outbreaks was also noticed in Sweden. The number of norovirus analyses sent to our laboratory increased fourfold

TABLE 4. Distribution of norovirus genetic clusters during 2000-2001, 2001-2002, and 2002-2003 seasons[a]

Genogroup	Variant[b]	No.					
		2000-2001[c]	2001-2002			2002-2003	
			Beginning	Peak	End	Beginning	Peak	End
I	GGI-2				1			
	GGI-3		1					
	GGI-4	2						
	GGI-6	2						
	GGI (not subtyped)					1		
II	GGII-1	3						
	GGII-2	2		1	1			
	GGII-4	29						
	GGII-4 (AATTTG motif)[d]		7	9	6			
	GGII-4 (AATCTG motif)[d]					7	10	10
	GGIIb[e]	30[f]			2	2		
	GGII (not subtyped)	18[g]						
IV	GGIV		1					
Not determined		1						

[a] A season was defined as starting in August and ending in July the following year.
[b] Classification according to reference 7.
[c] Reverse line blot hybridized (25).
[d] Data from reference 15.
[e] Accession number AJ487474.
[f] Nucleotide sequenced.
[g] One Rotterdam strain (GGII) and 17 unsubtyped GGII strains.

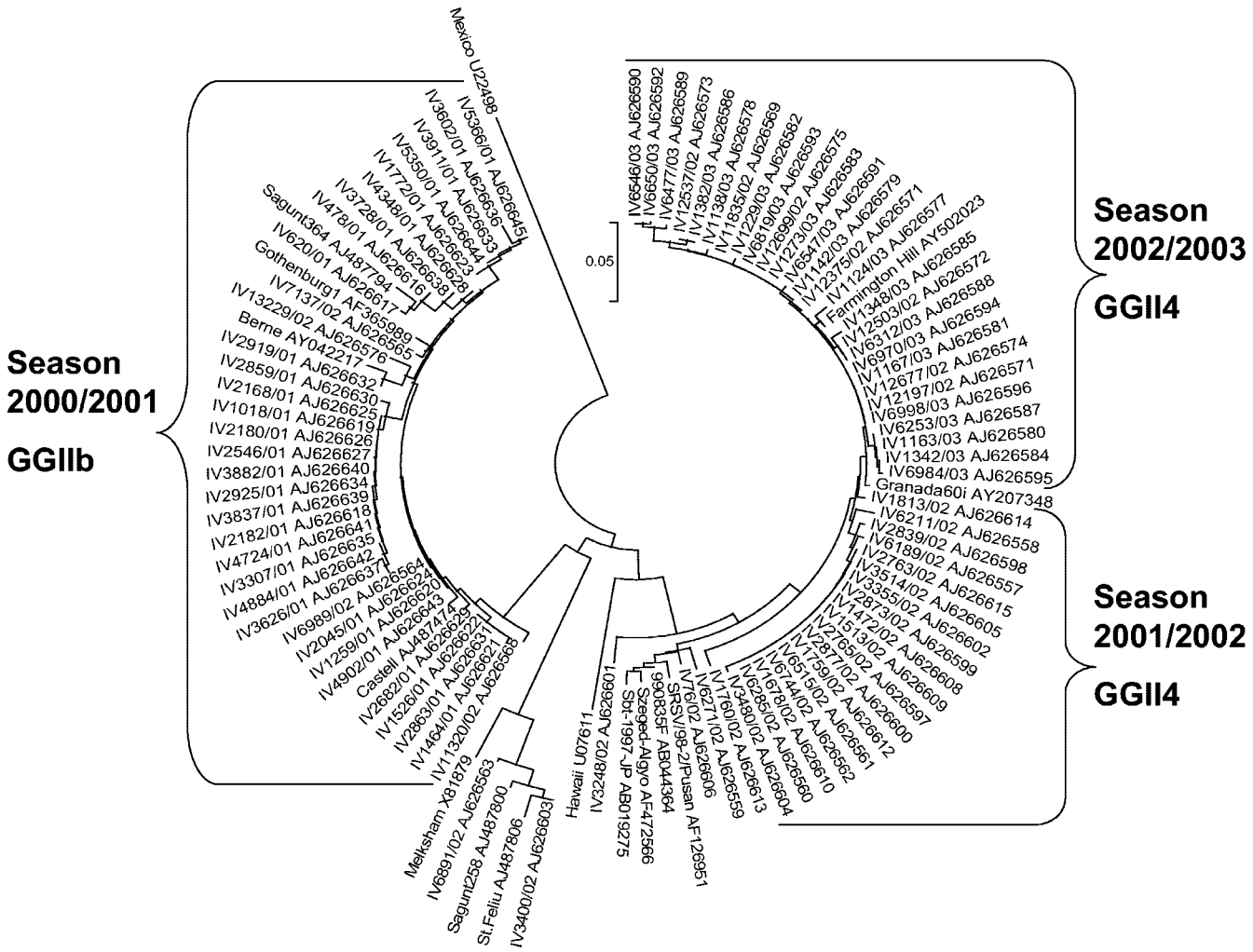

FIG. 3. Phylogenetic analysis of genogroup II norovirus strains from 2000-2001, 2001-2002, and 2002-2003 seasons. The phylogenetic tree was based on 310 nucleotides from the polymerase gene and was generated by the neighbor-joining method using Mega2.1 software. The strain identities precede the accession numbers.

from the first season to the third season, and the positive rate increased from 20 to 29% (Table 2). During the second and third seasons, the norovirus-positive specimens from children in relation to the total number of diagnosed patients dropped significantly from 47 to 9% and 5%, respectively (Fig. 2). This decline may be explained by the sudden rise in the new circulating variant, GGII-4, among adults in Sweden during the same period. The fact that the number of samples from children remained rather constant over the three seasons indicates that children might be less affected by the new GGII-4 variant than adults are.

RLB analysis is easy to handle and produces reliable results. It is based on oligonucleotides from established well-known norovirus types; however, emerging norovirus types cannot be identified by RLB. We failed to classify the GGII norovirus type in 47 specimens from the 2000-2001 season by RLB. Part of the RNA polymerase gene was sequenced in 30 strains. The 326-bp sequence, limited by the primer pair presented by Vinjé and Koopmans (24), showed 98 to 99% nucleotide homology to a norovirus strain described in outbreaks in Spain during 2001 (4). These viral strains clustered into a new variant designated GGIIb, which appeared to be related to the Mexico strain (4). This GGIIb type was the dominating strain circulating in Karolinska Hospital during the first season, especially among children (24 of 41 cases [59%]). This is probably an underestimate, since the 12 unsubtyped GGII strains among children may also belong to GGIIb. During the same season, Lordsdale-like noroviruses (GGII-4) were found in 29 of 86 (34%) positive patient specimens, and predominantly among adults (Table 3).

Interestingly enough, the GGIIb variant was also detected during the next two seasons, 2001-2002 and 2002-2003, in specimens from children. What is even more interesting is the decline of Lordsdale-like norovirus strains during the 2003-2004 season, while the GGIIb variant once again became the dominant norovirus type in norovirus-positive specimens from children (data not shown).

Almost 90% of the fecal specimens from children were obtained from the pediatric wards at Karolinska Hospital. The indications for taking fecal specimens from children with gas-

troenteritis have remained unchanged over the years. Most positive cases were found during the 2000-2001 season, when the GGIIb variant was circulating. As many as 18% of the pediatric specimens contained noroviruses compared with the overall positive rate of 20% (Table 2). This indicates that norovirus infections among children are as common as in adults. Further support for this is provided by reports from other parts of the world of a higher incidence of norovirus infections among children (6, 11, 18). In the last two seasons, the positive rates among children were 11 and 15%, respectively. Among the eight 2001-2002 and 2002-2003 season specimens from children that were nucleotide sequenced, four harbored GGIIb. This makes it tempting to hypothesize that GGIIb has a preference for children. This hypothesis is further supported by the findings from the 2003-2004 season, where there is a predominance of GGIIb among children (data not shown).

However, the association of GGIIb with children seen in the 2000-2001 season may also reflect a nosocomial spread of the strain in the pediatric wards during the first season. It should be pointed out that during that season we had a young child who was a long-time excretor of the same norovirus variant, GGIIb. She stayed in different pediatric wards in the hospital for two months. It is tempting to assume that this patient was the source of the GGIIb spread in the pediatric wards. However, the phylogenetic tree indicates that there are two clusters of GGIIb, which contradicts the hypothesis of nosocomial spreading (Fig. 3). The findings in our study might also reflect the community outbreaks of GGIIb. This is supported by findings from a recent publication in Sweden, where drinking water was contaminated by the GGIIb variant, causing a large gastroenteritis outbreak in 2001 (17).

During the three seasons, the dominant strain was a Lordsdale-like variant (GGII-4). The GGII-4 strains were found in 78 of the 145 (54%) genotyped norovirus-positive PCR specimens. No GGII-4 strains from the 2000-2001 season were nucleotide sequenced. The nucleotide sequence data from GGII-4 strains found in the 2001-2002 season showed 97 to 98% sequence homology to a strain that appeared in Japan as early as 1996 and has since been circulating there (11). In a recent publication by Lopman and coworkers (15), a compiled survey of norovirus outbreaks and molecular epidemiology in Europe from 1995 to 2003 was reported. The authors described a new variant of GGII-4 that appeared in 2002 as the cause of the majority of gastroenteritis outbreaks in Europe (15). This new variant, GGII-4, was described as having a characteristic set of mutations represented by the AATCTG motif in the polymerase gene (starting at position 4820 relative to Norwalk virus M87661). The association of this motif with the new GGII-4 variant was shown for a large number of strains from the beginning of 2002 (15). However, the motif was not recognized in our GGII-4 strains until the beginning of the third season studied (October 2002). One reason why we did not find this variant earlier might be the random selection of samples for nucleotide sequence analysis. Although the nucleotide sequences of the GGII-4 strains found during the 2001-2002 season were almost identical to the new variant, GGII-4, they contained a dissimilar motif, which was AAT TTG. This motif change is represented by only a single nucleotide mutation that seems to have appeared between the 2001-2002 and 2002-2003 seasons in the Stockholm region of Sweden. It is noteworthy that this nucleotide change does not significantly affect the clustering of the GGII-4 strains in the phylogenetic tree based on 310 nucleotides from the partial RNA polymerase region (Fig. 3). It may be questionable if a single nucleotide change in the polymerase gene is really significant, since the increased prevalence of GGII-4 had already started in the 2001-2002 season. To really confirm this new GGII-4 variant, there is a need to nucleotide sequence the capsid gene, where mutations most probably affect the norovirus phenotype. We believe that the GGII-4 strains found in Sweden in the second and third seasons are very similar in pathogenesis. The sudden increase in norovirus outbreaks started in the 2001-2002 season, although the new GGII-4 variant was not identified until the beginning of the 2002-2003 season (October 2002).

In summary, we have identified two dominant norovirus variants during the seasons studied. In the first season, the GGIIb variant was circulating, and in the second and third seasons, the new GGII-4 variant dominated. GGIIb seems to be closely associated with infections in children. This emphasizes the importance of further studies of the link between children and the GGIIb strain.

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