

Chronic Norovirus Infection after Kidney Transplantation: Molecular Evidence for Immune-Driven Viral Evolution

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Background. Norovirus infection is the most common cause of acute self-limiting gastroenteritis. Only 3 cases of chronic norovirus infection in adult solid organ transplant recipients have been reported thus far.

Methods. This case series describes 9 consecutive kidney allograft recipients with chronic norovirus infection with persistent virus shedding and intermittent diarrhea for a duration of 97–898 days. The follow-up includes clinical course, type of immunosuppression, and polymerase chain reaction for norovirus. Detailed molecular analyses of virus isolates from stool specimens over time were performed.

Results. The intensity of immunosuppression correlated with the diarrheal symptoms but not with viral shedding. Molecular analysis of virus strains from each patient revealed infection with different variants of GII.4 strains in 7 of 9 patients. Another 2 patients were infected with either the GII.7 or GII.17 strain. No molecular evidence for nosocomial transmission in our outpatient clinic was found. Capsid sequence alignments from follow-up specimens of 4 patients showed accumulation of mutations over time, resulting in amino acid changes predominantly in the P2 and P1–2 region. Up to 25 amino acid mutations were accumulated over a 683-day period in the patient with an 898-day shedding history.

Conclusion. Norovirus infection may persist in adult renal allograft recipients with or without clinical symptoms. No evidence for nosocomial transmission in adult renal allograft recipients was found in our study. Molecular analysis suggests continuous viral evolution in immunocompromised patients who are unable to clear this infection.

Noroviruses represent the most common cause of acute gastroenteritis in adults and older children worldwide. They belong to the family of the Caliciviridae, genus *Norovirus*. The first description was an outbreak in Norwalk reported in 1968, and the virus was named Norwalk virus thereafter. A wide range of genetically distant norovirus strains are organized into 5 genogroups and further classified into at least 27 genetic clusters or genotypes [1]. They are small (30 nm), single-stranded RNA viruses with a simple structure containing 1 major (VP1, capsid) and 1 minor (VP2) protein and no en-

velope. The genogroups GI, GII, and GIV include human pathogens, and the genotype II.4 has predominated in outbreaks, which were associated with epochal emergence of GII.4 variants [2–4].

The major route of transmission is fecal-oral when the patient is most symptomatic, but there is evidence of pre- and postsymptomatic transmission as well as occasional airborne transmission due to aerosolized viruses during vigorous emesis [5]. Noroviruses are extremely contagious and highly resistant to inactivation by freezing, heating, and exposure to detergent-based cleaners. The incubation period is short (24–48 h). The typical clinical presentation is an abrupt illness with vomiting and diarrhea, headache, or constitutional symptoms. Fever is present in one-half of patients. Symptoms generally last 24–60 h and are self-limited, but severe disease has been reported in elderly and immunocompromised patients.

Norovirus shedding in stool assessed by immune electron microscopy or antigen-capture enzyme-linked

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immunosorbent assay is rarely detected beyond 72 h after the onset of illness, but a prolonged shedding may be detected by using polymerase chain reaction (PCR) [6, 7]. It has been reported that levels of viral load in genogroup II infections is higher than in genogroup I [8]. In immunocompetent patients after experimental human infection virus, shedding up to 56 days has been described [9]. Siebenga et al [10] reported prolonged illness and viral shedding (21–182 days) in hospitalized patients. Chronic norovirus infection has been reported in pediatric intestinal transplant recipients and in a child with cartilage hair hypoplasia after bone marrow transplantation [11–13]. Ludwig et al [14] found a prolonged shedding over a maximum of 433 days in pediatric patients with cancer. Among adult solid organ transplant recipients, chronic excretion has only been reported in 1 patient undergoing heart transplant [15] and very recently in 2 renal transplant recipients [16]. Here, we describe a series of 9 consecutive cases of chronic norovirus infection in renal allograft recipients. To assess a potential nosocomial transmission and viral evolution over time, we performed a detailed molecular analysis, which excluded transmission in the outpatient clinic but revealed in-

creasing accumulation of mutations in the capsid gene over time.

PATIENTS AND METHODS

Patient screening and assessment. Renal transplant recipients presenting with prolonged or severe diarrhea were evaluated for infectious and noninfectious causes. In addition to routine assessment for bacterial or viral infection with stool cultures (for detection of *Campylobacter*, *Salmonella*, *Shigella*, and for patients with severe symptoms, protozoae), specific testing for *Clostridium difficile* toxin, and plasma PCR for cytomegalovirus, we performed PCR for norovirus from stool specimens. From November 2006 through November 2008, 78 patients were screened, and 13 (16.7%) were found to have positive PCR results for norovirus. If norovirus was detected, 2 additional PCR examinations were performed in the following 3 months, even if the patient had resolved all symptoms. Chronic norovirus infection was defined as 3 positive stool specimens during a follow-up of at least 3 months. Nine patients with viral shedding >3 months were found. In our 9 patients, over all 60

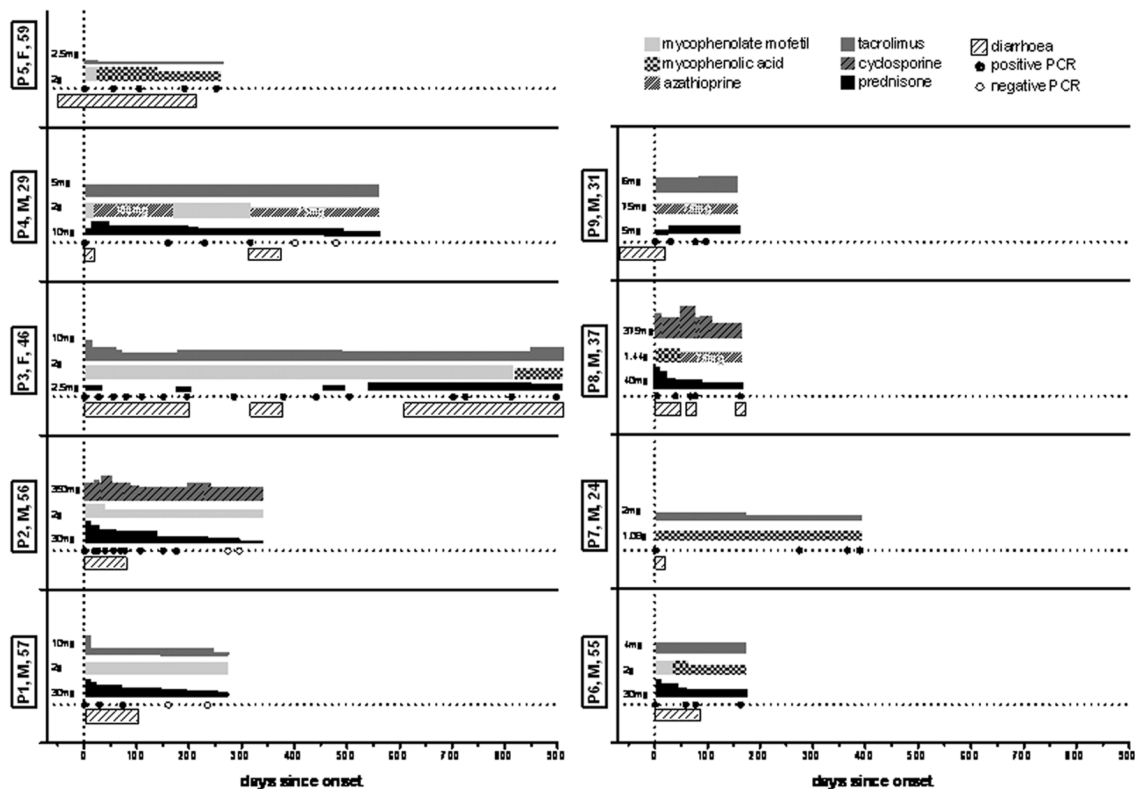


Figure 1. Correlation of immunosuppressive treatment with clinical symptoms and viral shedding. All patients received calcineurin inhibitor-based immunosuppression. Chronic norovirus shedding was observed over 97–898 days. The occurrence of clinical symptoms but not C-reactive protein levels and viral shedding correlated with the intensity of immunosuppression. F, female; M, male; P1–P9, patients 1–9. The number after sex indicates the patient’s age.

samples were analyzed (mean, 6.67 samples tested per patient). Figure 1 shows sample numbers in chronological sequence for each patient. Clinical symptoms, immunosuppression dose, and C-reactive protein levels were recorded at every visit. Furthermore, 7 of our 9 patients underwent colonoscopy and gastroscopy. In case of persistent norovirus infection during the first 3 months after initial diagnosis, PCR for norovirus was regularly performed every 3 months thereafter and was only stopped after 2 consecutive negative results. Duration of shedding was defined by the dates of first positive and first negative PCR results. Family members were not tested.

PCR and molecular analysis. Analysis of stool specimens for the presence of norovirus was performed at the Institute of Medical Virology, University of Zürich, Switzerland. Stool specimens were diluted 10-fold with phosphate-buffered saline, were frozen over night at -20°C , and after thawing, were centrifuged in a table top centrifuge at 1000 g for 15 min. On the basis of threshold cycle values of the real time PCR, we divided positive specimens into groups with very high, high, and average/low virus concentration. Diagnostic PCR for norovirus genogroup I and II were performed as described by Höhne and Schreier [17].

To analyze the polymerase genotype, a 297-basepair fragment of region A [18] was amplified from the first available stool sample of all 9 patients and sequenced directly as described elsewhere [19]. The accumulation of mutations in the capsid gene was analyzed in follow-up samples obtained from 4 patients (patients 3, 4, 5, and 7). Therefore, the entire ORF2 gene region was reverse transcribed and amplified in the first round reverse transcription PCR using SuperScript III One-Step RT-PCR System with Platinum Taq High Fidelity, according to the manufacturer's instructions (Invitrogen), with primers NV107a (sense, 5'-AGCCAATGTTTCAGATGGATG-3') and NV100 (antisense, 5'-GCAAAGAAAGCCTCCAGCCAT-3'). The 1-step reverse transcription PCR was performed at 55°C for 5 min, 45°C for 55 min, and 94°C for 2 min, followed by 40 cycles at 94°C for 15 s, 45°C for 30 s, and 68°C for 2 min, and finally, a 5 min elongation at 68°C . For the nested PCR, primers NV271 (sense, 5'-ATGAAGATGGCGTCTGAATGA-3') and NV288 (antisense, 5'-TAAAGCACGCCTGCGCCCCG-3') and Platinum Pfx DNA polymerase (Invitrogen) were used. Amplicons were purified from agarose gels with use of the MinElute Gel Extraction Kit (Qiagen), or from solution with use of ExoSap-It (GE Healthcare). Purified amplicons were sequenced directly using the BigDye terminator cycle sequencing kit and an ABI 3130xl Genetic Analyzer (Applied Biosystems). Sequences were aligned to prototype sequences drawn from GenBank with use of CLUSTAL W, version 1.6, and phylogenetic trees were produced using the neighbor joining and DNADIST program of the Phylogeny Interference Package (PHYLIP), version 3.57c.

Table 1. Patient Characteristics

Characteristic	All patients (n = 9)
Sex	
Female	2 (22)
Male	7 (78)
Age, median years (range)	46 (23–59)
Renal disease	
Glomerulonephritis	3 (33)
Hypoplasia/ Aplasia	3 (33)
Others	3 (33)
Duration of dialysis, median months (range)	29 (3–136)
Hemodialysis	5 (56)
Peritoneal dialysis	4 (44)
Transplantation	
Prior transplantations, median no (range)	1 (1–2)
Living donor kidney	3 (33)
Deceased donor kidney	6 (67)
Induction therapy	2 (22)
Rejections, median no (range)	1 (1–2)
Rejection treatment	
Prednisone	6 (67)
ATG/OKT3	2 (22)
Immunoadsorption	1 (11)
Initial immunosuppression	
Dual immunosuppression	
With CyA	0 (0)
With tacrolimus	2 (22)
Triple immunosuppression	
With CyA	2 (22)
With tacrolimus	5 (56)
Antimetabolite	
Mycophenolate mofetil	6 (67)
Mycophenolic acid	2 (22)
Azathioprine	1 (11)
Prednisone	7 (78)
Norovirus infection	
Start after last transplantation, median months (range)	42 (1.3–136)
CRP level at initial presentation, median mg/L (range)	1 (1–38)
Need for patient hospitalization	5 (56)
Duration of symptoms, median days (range)	150 (24–898)
Intermittent symptoms	5 (56)
Duration of shedding, median days (range)	230 (97–898)
Viral clearance	3 (33)

NOTE. Data are no (%) of patients, unless otherwise indicated. CRP, C-reactive protein; CyA, cyclosporin A.

Nucleotide sequences of the capsid gene from the first and the last specimen amplified were submitted to GenBank (accession numbers, GQ266690–GQ266697).



Figure 2. Sequence analysis of a polymerase gene fragment from all 9 patients. Neighbor-joining tree of a 297 nucleotide– long fragment of the polymerase gene (region A). Sequences were obtained from the first stool specimens available for all 9 patients (P1–P9). Prototype sequences (*bold, italics*) from GenBank are II.1 Hawaii (U07611), II.3 Toronto (U02030), II.4 2006A Terneuzen70 (EF126964), II.4 2006B Nijmegen115 (EF126966), II.7 Leeds (AJ277608), and II.17 Sommieres1203/2006 (EF529742). Bootstrap values >70% are indicated. The scale represents nucleotide substitutions per site.

RESULTS

Patient characteristics. Nine consecutive patients (7 men and 2 women) with a renal allograft and gastroenteritis due to chronic norovirus infection were identified from November 2006 through November 2008 and were followed up until April 2009. The age of the patients at onset was 23–59 years. Norovirus infection started between 1.3–125 months after transplantation (median, 42 months). At the onset of norovirus infection, 7 patients were receiving standard triple immunosuppression with a calcineurin inhibitor, mycophenolate mofetil, and prednisone; 2 patients were receiving dual immunosuppression with a calcineurin inhibitor and mycophenolate mofetil; 7 patients were receiving tacrolimus; and 2 patients were receiving cyclosporine A (Table 1).

Clinical course of norovirus infection. Duration of chronic virus shedding ranged from 97 to 898 days (Figure 1). A very high virus concentration (threshold cycle value, 15–20; mean, 17.29) was found in 18.4%, and a high concentration (threshold cycle value, >20–30; mean, 24.22) was in 71.4% of all positive specimens. In 10.2% of samples with positive PCR results for norovirus, the concentration was low to average (threshold cycle value, >30–38; mean, 34.45); in most of these, the following PCR for norovirus had a negative result. In all cases, diarrhea led to a reversible prerenal decrease of renal function.

Five patients were hospitalized because of severe dehydration and allograft dysfunction. Symptoms of overt diarrhea, irregularly formed stools, and/or abdominal bloating and pain in repeated consultations lasted 24–898 days. Patient 3 had the longest follow-up, with chronic viral shedding over 898 days, and intermittent clinical symptoms were documented over the whole study period after detection of the first positive stool. At initial presentation, C-reactive protein was mildly elevated (median, 1 mg/L; range, 1–38 mg/L), whereas in the asymptomatic shedding phase, the C-reactive protein level remained normal except for episodes associated with other infections. Three of the 9 patients cleared the virus during the observation period after 104–379 days. Because of the lack of a specific therapy, a cautious decrease of immunosuppression was initiated if possible, mainly by reduction or withdrawal of prednisone or mycophenolate mofetil. Two patients were switched from mycophenolate mofetil to azathioprine because it is associated with fewer gastrointestinal adverse effects. Reduction of immunosuppression led to clinical amelioration or full recovery in all patients, but norovirus shedding only stopped in 3 patients.

Results of sequence analysis I: no evidence for nosocomial transmission. Sequence analysis of region A in the polymerase gene (Figure 2) and region C (5' end of the capsid gene; data

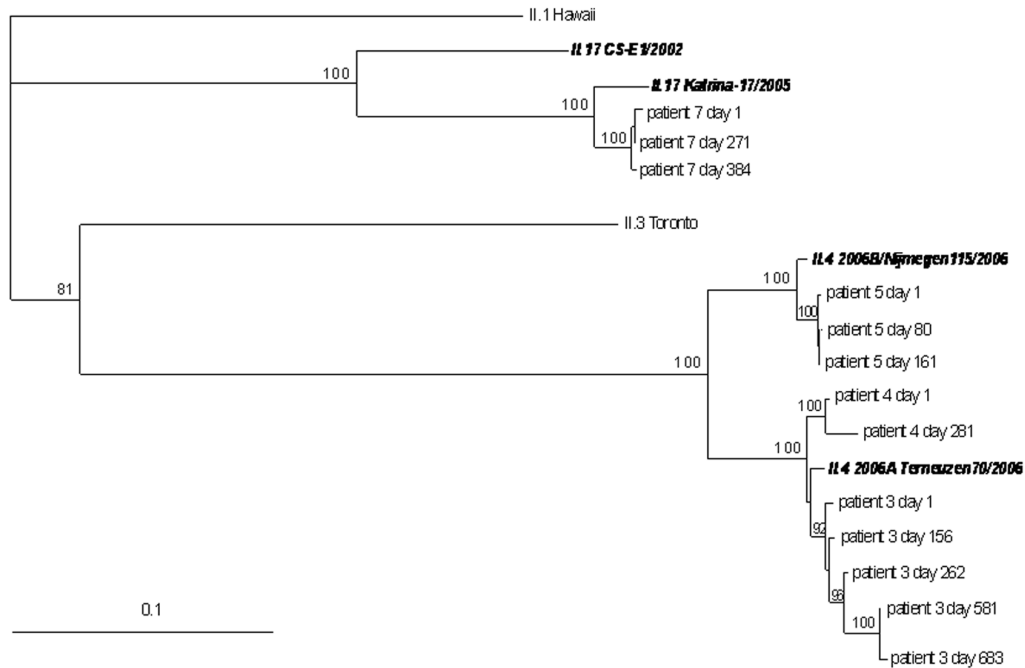


Figure 3. Sequence analysis of the capsid gene in follow-up samples from 4 patients. Neighbor-joining tree based on alignments of 1596 nucleotide-long fragments of capsid gene sequences from patients 3, 4, 5, and 7. Prototype sequences (*bold, italics*) from GenBank are II.1 Hawaii (U07611), II.3 Toronto (U02030), II.4 2006A Terneuzen70 (EF126964), II.4 2006B Nijmegen115 (EF126966), II.17 CS-E1/2002 (AY502009), and II.17 Katrina-17/2005 (DQ438972). Bootstrap values >70% are indicated. The scale represents nucleotide substitutions per site.

not shown) revealed that patients 2, 3, 4, and 8 were infected by GII.4 variant 2006A (prototype sequence Terneuzen/70/2006; GenBank accession number EF126964), and patients 1, 5, and 9 were infected by GII.4 variant 2006B (prototype sequence Nijmegen115/2006; GenBank accession number EF126966), with DNA distances of 0.0102–0.0345 among each other. Furthermore, comparison of nearly full-length capsid sequences of the earliest samples available from patients 2, 3, and 4 (all infected with GII.4 2006A) demonstrated 1.7%–2.4% differences in nucleic acid and 1%–1.5% differences in amino acid sequences. In patients 6 and 7, the non-GII.4 genotypes GII.7 and GII.17 were detected, respectively. All patients remained infected by the initially detected strain throughout the period of the study. Because of the finding of 3 different genotypes and 2 variants of GII.4, no evidence for nosocomial transmission in the outpatient clinic was found for the 9 patients analyzed.

Results of sequence analysis II: evidence for viral evolution.

To study viral evolution, nearly full-length sequences of the capsid gene (1596 nucleotides long) in follow-up samples from 4 patients (patients 3, 4, 5, and 7) were analyzed, and the neighbor-joining tree is shown in Figure 3. In 5 serial stool specimens from patient 3 (infected by GII.4 2006A; shedding over 898 days) obtained over a period of 683 days, 46 nucleotide changes occurred, resulting in 25 amino acid changes. The accumulation and fixation of amino acid changes resulted in

an overall fixation rate of 0.037 amino acids/day. Interestingly, a biphasic pattern with a decreased fixation rate after day 262 was observed (0.049 before day 262 and 0.027 after day 262), which may be related to a reduction of immunosuppressive therapy (ie, stop of prednisone; Figure 4). Twenty-one of 25 amino acid changes were accumulated in the P2 and P1–2 domain (amino acids 294–459), including amino acid substi-

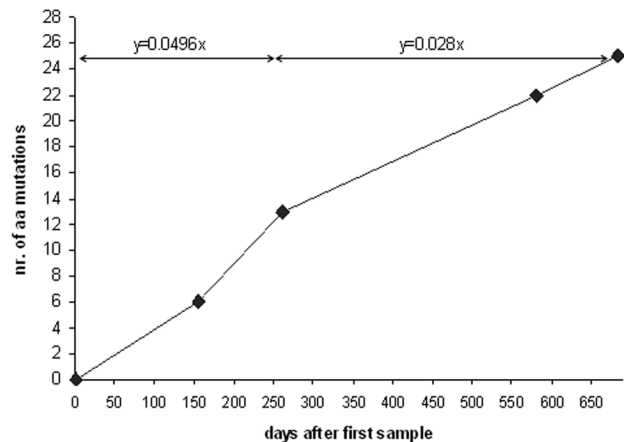


Figure 4. Rate of amino acid change fixation of norovirus recovered from patient 3. During the first 262 days of chronic norovirus infection, an accumulation of 0.049 amino acid changes/day occurred, compared with 0.028 amino acid changes/day during days 262–683.

		site A												site B												
		N	S	P1-1				P2				P1-2														
		AA position																								
patient 3	day	17	98	234	268	294	296	297	298	327	340	348	356	357	372	382	391	393	404	407	412	413	425	426	456	459
	1	N	S	V	T	A	T	Q	E	V	R	K	V	H	S	R	D	G	V	N	D	G	S	F	E	Q
	156	N	S	V	T	A	T	Q	V	V	R	K	A	D	S	R	D	S	V	D	D	V	S	F	E	Q
	262	S	S	V	I	E	T	Q	V	V	R	K	A	E	S	R	D	S	V	D	G	V	T	Y	D	Q
	581	S	G	I	I	E	S	Q	V	K	S	K	A	E	N	K	N	S	E	D	G	I	T	Y	D	Q
	683	S	G	I	I	E	S	K	V	K	S	T	A	E	N	K	N	S	E	D	G	I	T	Y	D	R
patient 4	6			256		295		297	298		340				357			389	393	404	411	412			450	521
	1	S		A		G		Q	E		R			Q			V	S	V	R	D				D	V
	281	N		T		S		R	V		G			E			I	N	A	S	G				E	I
patient 5											340							393								
	1										G							S								
	80										R							N								
	161										R							N								
patient 7			193			295								358	378	383			401							
	1		D			Q								A	E	G			F							
	271		E			Q								A	D	G			F							
	384		E			H								T	D	S			C							

Figure 5. Amino acid substitutions in the capsid gene during long-term shedding from patients 3, 4, 5, and 7. Capsid domains are indicated in the first bar. Changing amino acid positions are shown at the top of the 1-letter amino acid code. *Gray boxes* indicate informative sites according to Siebenga et al [10], and *asterisks* indicate hot spots according to Allen et al [20]. Labeled bars indicate amino acid positions belonging to antigenic sites A and B.

tutions at positions 296–298 and 393, which are considered to be sites putatively associated with antigenic changes (site A, amino acids 296–298; site B, amino acids 393–395) [20]. In patient 4, who was also infected by GII.4 2006A, 22 nucleotide changes resulting in 14 amino acid changes were found after 281 days (0.049 amino acid changes/day). Twelve amino acid changes occurred in the P1 and P2 domain positions 256 and 450, and 1 each occurred in the extreme N- and C-termini of the capsid protein (amino acid 6 and amino acid 521, respectively). Also in this patient, amino acid substitutions occurred in site A and B. The 2 other patients demonstrated more reduced fixation rates. Patient 5 (infected by GII.4 2006b) had a fixation rate of 0.012 amino acid changes/day (2 amino acid changes, 3 nucleotide changes) after 161 days of infection, and in patient 7, (infected by GII.17) a fixation rate of 0.013 amino acids/day was observed after 384 days of chronic infection (6 amino acid changes, 10 nucleotide changes). All amino acid changes observed in the capsid gene of the 4 patients are represented in Figure 5.

DISCUSSION

This study presents a detailed clinical and molecular analysis of a series of 9 adult renal allograft recipients with chronic norovirus infection. This infection in immunocompetent patients is usually self-limiting and of short duration. Virus shedding 13–56 days after inoculation has been described in experimentally infected volunteers [9]. Kirkwood [21] investigated 8 children recovering from norovirus gastroenteritis and demonstrated a shedding for at least 25 days in 3 children and up to 100 days in 1 child. Prolonged asymptomatic shedding has been reported in special groups of immunocompromised

patients, such as small children or geriatric hospitalized individuals [10, 22]. In adult solid organ transplant recipients, chronic excretion has been reported in 1 heart transplant patient [15]. Westhoff et al [16] found virus shedding in 2 kidney allograft recipients over a maximum of 7.5 months. In our series, we found chronic virus shedding in 9 kidney allograft recipients with a median shedding duration of 230 days and a maximum of 898 days. Clinical symptoms lasted 24–898 days. Initial clinical presentation was acute gastroenteritis, as expected, whereas further clinical follow-up was more heterogeneous. Although no specific therapy for this infection is available, intravenous fluid and electrolyte substitution and hospitalization was required in severe cases. Reduction of immunosuppression may be indicated in situations with severe symptoms and chronic shedding. Adjustment of immunosuppression needs to be performed with great care and must be individualized for each patient. Blood levels of calcineurin inhibitors, in particular tacrolimus, tend to increase during symptomatic episodes and should be adjusted [23, 24]. Intensity and duration of symptoms as well as virus shedding were influenced by dosage reductions of steroids or mycophenolate mofetil or by a switch from mycophenolate mofetil to azathioprine. Because we did not alter tacrolimus or cyclosporine treatment, no conclusions can be made with regard to the role of calcineurin inhibitors. Interestingly, after reduction of immunosuppression most recipients were able to recover completely despite chronic virus shedding after a phase of stool irregularities (loose stool without diarrhea or meteorism). Thus, in renal allograft recipients presenting with diarrhea, norovirus should be included in the differential diagnosis. PCR for norovirus in

stool samples is routinely available and should be included in the work-up of these patients.

Molecular analysis of norovirus isolates, including follow-up specimens, was performed with the following 2 aims: (1) addressing a potential nosocomial transmission and (2) evaluating virus evolution over time under a reduced immune pressure due to maintenance immunosuppressive treatment. Contamination of environmental surfaces, such as sanitary equipment, scales, or objects in the examination room, is known to play a role in norovirus transmission [25, 26]. Therefore, hospitalized symptomatic patients with proven norovirus infection are usually isolated. However, in the outpatient setting with chronically infected patients the situation is less clear. Recently, Xerry et al [27] demonstrated that a point source outbreak is characterized by 100% nucleotide similarity in the capsid P2 domain among strains from different patients. In contrast, in our 9 patients 3 different norovirus genotypes were detected. Furthermore, among the 7 patients infected with genotype II.4, 2 variants (2006A and 2006B) differing 1%–6% in the nucleotide sequences of the capsid region were identified. Thus, although in our outpatient clinic all renal transplant recipients share a common waiting area and use the same toilets, the molecular analysis of virus strains showed no evidence of nosocomial transmission. This surprising observation may be explained by a lower amount of virus shedding and/or a lower infectiousness of mutated viruses in chronically infected patients, compared with acute norovirus infection. However, this has not yet been proven, and therefore, the exact role of contact isolation in asymptomatic and symptomatic solid organ recipients in the outpatient clinic setting still needs to be defined.

The second goal of molecular analysis was to assess viral evolution in chronically infected patients shedding norovirus over several months. By comparison of subsequent epidemic variants of GII.4 strains, the existence of several hotspots of amino acid variation across the P domain has been demonstrated [2, 3, 28]. Analysis of mutations at these hotspots and their mapping onto the 3D crystal structure revealed 2 surface-exposed sites in the P2 domain (site A and site B, both 3 amino acid residues in length) which were strongly associated with the emergence of epidemiologically distinct virus strains [20]. Binding studies using monoclonal antibodies confirmed that site A and B form a conformational, variant-specific epitope which is involved in antibody binding, possibly leading to escape mutants [29]. In all 3 GII.4-infected patients in this study, most of amino acid changes occurred within the hotspots described for the emergence of new variants. In patient 3, with an 898-day shedding history, 14 of 17 amino acid substitutions occurred among amino acid residues 294 and 425, including amino acid changes at all 3 positions of site A (amino acid residues 296–298) and 1 amino acid substitution at site B (amino

acid 393). In contrast, all 6 amino acid mutations found in patient 7, who was infected with GII.17, occurred outside the hotspots described for GII.4 strains. On the basis of crystal structure analysis of a Lordsdal-like GII.4 P protein (VA387 strain), Cao et al [28] located the receptor binding pocket responsible for binding to α -fucose of the histoblood group antigens at the outermost end of the P domain (amino acid residues Ser-343, Thr-344, Arg-345, Asp-374, Cys-441, Ser-442, and Gly-443). In all of our 4 patients analyzed, no amino acid substitutions at these sites were observed, but in all 3 GII.4-infected patients, the amino acid at position 340 was changed, which is close to the receptor binding site (amino acids 343–345). Donaldson et al [30] suggested that just a few amino acid changes within the P2/P1 domain can influence the antigenicity and the receptor usage, leading to an increasing ability of the virus to persist. The relatively low amino acid accumulation rates of our patients, which seem to be attributable to their impaired immunity, confirm the data reported by Siebenga et al [10], who studied patients of different levels of immunodeficiency. Patients with highly or mildly impaired immune response demonstrated a fixation rate of 0.03 and 0.07 amino acid changes per day, respectively, whereas 0.13 amino acid changes per day were found in an otherwise healthy patient. Thus, amino acid changes within the P2 surface also detected in solid organ allograft recipients receiving maintenance immunosuppression showed that even a weak immune pressure causes modification of the capsid protein to evade immune recognition, which may be part of the persistence strategy of norovirus.

Taken together, to our knowledge, this study for the first time presents a series of renal allograft recipients with chronic norovirus infection, including a molecular analysis of norovirus isolates. Norovirus infection in immunosuppressed patients can occur for up to 898 days with or without clinical symptoms. The implications for hospital hygiene procedures in inpatients and outpatients have to be defined. Molecular analysis demonstrated no evidence of nosocomial transmission in our outpatient clinic but suggested immunity-driven virus evolution.

Acknowledgments

Potential conflicts of interest. All authors: no conflicts.

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