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Short communication

Super-infections and relapses occur in chronic norovirus infections

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ABSTRACT

Background: Norovirus causes chronic infections in immunocompromised patients with considerable associated morbidity. It is not known whether chronic infections involve super- or re-infections or relapses.
Objectives: To retrospectively investigate whether longitudinal sampling in chronically infected patients demonstrates persistent infection with the same virus, or super- or re-infection.
Study design: Norovirus full genomes were generated from 86 longitudinal samples from 25 paediatric patients. Consensus sequences were used for phylogenetic analysis and genotyping.
Results: Super-infections occurred in 17% of chronically infected patients who were continuously PCR positive; including two with mixed norovirus infections. The median duration of infection was 107 days longer in those with super-infections; however this was not statistically significant. A third of patients with interrupted norovirus shedding continued to be infected with the same virus despite up to 2 months of PCR negative stools, classified as a relapse. The majority (67%) of patients with interrupted shedding were re-infected with a different genotype.
Conclusions: Chronically infected patients who are continuously PCR positive are most likely to remain infected with the same virus; however super-infections do occur leading to mixed infection. Patients with interrupted shedding are likely to represent re-infection with a different genotype, however relapsing infections also occur.

Our findings have implications for infection control as immunosuppressed patients remain susceptible to new norovirus infections despite current or recent infection and may continue to be infectious after norovirus is undetectable in stool. The relevance to children without co-morbidities remains to be determined.

1. Background

Norovirus is a leading cause of gastroenteritis. Infections are typically self-limiting in immunocompetent hosts, with limited morbidity aside from dehydration. In immunocompromised patients however, there is a risk of chronic infection with significant associated morbidity [1]. Chronic infections are bi-phasic with an acute phase of vomiting and diarrhoea, followed by chronic viral shedding and diarrhoea lasting weeks to years. The majority of case reports describe patients to be symptomatic during this extended period of shedding, with up to 24 bowel movements per day [2]. However chronic infections can experience intermittent symptoms of diarrhoea [3] or be asymptomatic [4].

The *Norovirus* genus is comprised of five genogroups (GI–GV), of which GI, GII and, to a limited extent, GIV cause infections in humans. Each genogroup is further classified into genotypes; GI.1–9 and GII.1–22. GII.4 genotypes, which are the predominant global genotype since the mid-1990s [5], are divided into variant types. Norovirus has a dual typing system based on the polymerase (ORF1) and capsid (ORF2) sequences.

2. Objectives

We retrospectively sequence full norovirus genomes from longitudinally sampled chronic infections for genotyping and phylogenetic analysis, to determine whether patients remain persistently infected with the same strain or whether super- or re-infections occur.

3. Study design

Eighty-six longitudinal stool samples were retrospectively sequenced from 25 paediatric patients, with two to eight samples per patient. Samples were collected between November 2012 and January 2016 from patients with persistent norovirus infections (PCR positive > 1 month) for whom two or more longitudinal stool specimens were available. Patients were under the care of a UK paediatric tertiary

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referral hospital. Norovirus positive patients were tested weekly whilst inpatients or monthly whilst outpatients for the presence or absence of norovirus by the diagnostic Virology laboratory using a reverse-transcriptase real-time multiplex PCR to detect norovirus GI and GII, the methods for which are described elsewhere [6].

Of the 25 patients, 18 were continuously norovirus positive (continuous shedding), with a median of 129 days between the first and last sequenced sample (range 7–466). A further seven patients had a period between the first and last sequenced sample during which norovirus was not detected in stool, before once again being detected (interrupted shedding). In addition two of the 18 patients who shed norovirus continuously (Patient 63 and 73), proceeded to become norovirus PCR negative following which both became positive again. In total nine patients had interrupted norovirus shedding (median 153 days undetected, range 9–466).

Norovirus genome sequencing and phylogenetic analysis are described in Supplementary Methods.

4. Results

4.1. Continuously positive patients

Of the 18 patients who were continuously norovirus PCR positive, 15/18 (83%) remained infected with the same genotype throughout the study period, classified as persistent infections (Table 1). The longitudinal samples from each of these patients cluster together on the phylogenetic tree (Fig. 1), indicating these patients remained infected not only with the same genotype but with the same virus.

Three of the 18 (17%) patients with continuous shedding had evidence of infection with a second genotype occurring during the study period (Patients 73, 65 and 101), classified as super-infections. Super-infection is proven for two patients (Patient 65 and Patient 101) in whom co-infection with two different genotypes was detected in a single sample. Patient 73 was initially infected with a GII.Pe_GII.4 virus then became infected with GII.P16_GII.17, although a mixture of the two genotypes in the same sample was not detected. Patient 73 was continually positive for norovirus in stool; the interval between detection of GII.Pe_GII.4 and of GII.P16_GII.17 was 22 days with an additional positive stool sample taken during this interval (not available for sequencing). We cannot confirm whether Patient 73 cleared GII.Pe_GII.4 prior to infection with GII.P16_GII.17 or whether a temporary mixed infection occurred, however given the short interval between positive PCR tests (1–2 weeks), the latter is most probable.

These data suggest that in patients who are continuously norovirus PCR positive, super-infection occurs in a sixth (17%) of cases. The median duration of infection was 322 days (range 58–738 days) in the three patients who had a super-infection and 215 days (range 14–711 days) in the 15 who did not. The duration of infection was not significantly different ($P = 0.360$).

4.2. Patients with interrupted norovirus shedding

Of the nine patients who become norovirus PCR negative and then positive again, five (Patients 34, 68, 73, 147 and 176) acquired a second virus with a different genotype to the first, classified as re-infection (Table 1). For Patient 73 this was the second incidence of re-infection, the first having occurred whilst continuously norovirus PCR positive (Supplementary Fig. 1).

Another of the nine patients, Patient 63, appeared to be infected with the same genotype (GII.P21_GII.3) after a period of 466 days during which norovirus was undetectable by PCR. Phylogenetic analysis revealed the second virus to be a different variant of GII.P21_GII.3, since the sequences from before and after the PCR negative period do not cluster together (Fig. 1).

Thus the majority (6/9, 67%) of patients with interrupted norovirus shedding had been re-infected with a different genotype or variant.

For the remaining three patients (Patients 31, 72 and 75), the second virus was of the same genotype, clustering with the earlier virus in the phylogenetic analysis tree (Fig. 1), classified as relapse. This suggests cryptogenic persistence of the first virus. The three relapse patients had the shortest intervals during which norovirus was undetectable; less than two months compared to 2–15 months for those who were re-infected with a new genotype or variant.

4.3. Single nucleotide variants in longitudinal samples

Excluding those patients with mixed infections, in the patients who were continually infected with the same virus there was a strong positive correlation between the number of consensus sequence pairwise single nucleotide variants (SNVs) and the number of days separating specimen collection (R^2 0.775, $P < 0.001$) (Supplementary Fig. 2) with up to 131 SNVs accumulating across the genome over 445 days.

5. Discussion

We use full genome sequencing to show that super-infection and re-infection occurs in patients in whom norovirus can be detected over long periods. When the virus is shed continuously, super-infection was detected in a sixth (17%) of patients while re-infections accounted for the majority (67%) of cases where norovirus was detected after interrupted shedding. Whether a lack of protection against super- and re-infection extends to children without comorbidities remains to be determined.

Conversely, relapse was identified in patients in whom norovirus shedding was interrupted for up to two months. These data may have implications for clinical practice; chronically infected patients who appear to clear norovirus may still harbour persistent but undetectable virus. Whether or not these patients present a transmission risk is not known. However, a prudent course of action would be to consider immunosuppressed patients who have cleared virus after a chronic infection as potentially infectious for up to 2–3 months following the last positive stool and to continue PCR surveillance for this period. Given the small sample size in this study (three patients relapsing) a larger study is required to confirm these findings.

Our data confirms previous observations that viruses persistently infecting immunocompromised patients are continuously mutating, leading to the accumulation of SNVs [3,7]. The resulting intra-host population can be observed as a heterogeneous quasispecies which some have suggested may be a reservoir for the emergence of novel viral variants [7,8], however the estimated rarity of such events has led to the conclusion that immunosuppressed hosts are not the principle source of novel variants at the epidemiological scale [9].

Mixtures of norovirus strains have been detected in individuals in oyster-borne norovirus outbreaks [10,11]; to our knowledge this is the first identification of mixed genotypes within a single host in sporadic infections. Co-infecting norovirus strains within an individual provides the opportunity for viral recombination to occur, a feature that is known to be important in norovirus evolution and has been suggested to contribute to the emergence of new pandemic strains [12].

Conflict of interest statement

The authors declare no conflict of interest. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

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Table 1
Summary of longitudinally sampled norovirus infections. The occurrence of re-infection is inferred from phylogenetic analysis of norovirus full genome sequences.

Patient ID	Age	Comorbidity	Number of sequenced samples	Days between first and last sequenced sample	Interrupted shedding**	Original genotype	Re-infecting genotype	Classification of chronic infection †
101	3 years	BMT	2	466	No	GII.P21_GII.3	mixed GII.P21_GII.3/ GII.P7_GII.6	Super-infection
50	1 year	Heart transplant	2	446	No	GII.Pe_GII.4 Sydney_2012	–	Persistence
58	1 year	BMT	2	445	No	GII.P4_GII.4 Sydney 2012	–	Persistence
65	2 years	HSCT	5	321	No	GII.P21_GII.3	mixed GII.P21_GII.3/ GII.Pe_GII.4	Super-infection
64	11 years	BMT	2	243	No	GII.P4_GII.4 New Orleans 2009	–	Persistence
67	1 year	BMT	7	197	No	GII.P21_GII.3	–	Persistence
177	9 months	BMT	2	190	No	GII.P21_GII.3	–	Persistence
174	1 year	BMT	2	163	No	GII.P7_GII.6	–	Persistence
61	13 years	BMT	5	123	No	GII.P21_GII.3	–	Persistence
66	3 years	Lung transplant	2	35	No	GII.P7_GII.6	–	Persistence
74	1 year	Polycystic renal disease	4	28	No	GII.P21_GII.3	–	Persistence
71	6 years	Inflammatory disorder	3	14	No	GII.P21_GII.3	–	Persistence
70	5 months	Oropharyngeal dysphagia	4	13	No	GII.P21_GII.3	–	Persistence
62	3 months	HSCT	2	7	No	GII.P4_GII.4 New Orleans_2009	–	Persistence
54	2 years	BMT	2	7	No	GII.Pe_GII.4 Sydney_2012	–	Persistence
40	5 months	SCID	2	7	No	GII.Pe_GII.4 Sydney_2012	–	Persistence
63	1 year	Leukaemia	3	1006	No	GII.P21_GII.3	–	Persistence
					Yes (466 days, 29 samples)	GII.P21_GII.3	GII.P21_GII.3	Re-infection
73	2 years	BMT	6	156	No *	GII.Pe_GII.4 Sydney_2012	GII.P16_GII.17	Super-infection
					Yes (57 days, 5 samples) *	GII.P16_GII.17	GII.P21_GII.3	Re-infection
147	10 years	Leukaemia	2	339	Yes (11 months, 10 samples)	GII.P4_GII.4 New Orleans_2009	GII.P21_GII.3	Re-infection
68	10 years	Leukaemia	5	202	Yes (6 months, 13 samples)	GII.P4_GII.4 Sydney 2012	GII.P7_GII.6	Re-infection
34	4 years	BMT	2	178	Yes (5 months, 2 samples)	GII.P21_GII.3	GII.Pe_GII.2	Re-infection
176	3 years	Cardiomyopathy	2	157	Yes (5 months, 10 samples)	GII.P2_GII.2	GII.P7_GII.6	Re-infection
31	1 year	SCID	7	123	Yes (56 days, 6 samples)	GII.Pe_GII.4 Sydney 2012	–	Relapse
72	1 year	SCID	5	73	Yes (23 days, 1 sample)	GII.P21_GII.3	–	Relapse
75	1 year	BMT	6	62	Yes (9 days, 1 sample)	GII.P7_GII.6	–	Relapse

BMT, bone marrow transplant; HSCT, hematopoietic stem cell transplant; SCID, severe combined immunodeficiency.
- unchanged from original.

† Persistence, persistent infection with the same virus with continuous shedding; Relapse, persistent infection with the same virus after a period of interrupted shedding (PCR negative in between sequenced samples); Super-infection, infection with a new virus whilst already norovirus positive; Re-infection, infection with a new virus following a period of interrupted shedding (PCR negative in between sequenced samples).

* Patient 73 was re-infected twice; the first (GII.Pe_GII.4 Sydney_2012 to GII.P16_GII.17) was whilst the patient was continuously norovirus PCR positive (super-infection) and the second (GII.P16_GII.17 to GII.P21_GII.3) was after a period of 57 days during which norovirus was undetectable by PCR (re-infection).

** Interrupted shedding is defined as a period between the first and last sequenced sample during which norovirus was not detected in stool, before once again being detected. The number of days or months shown is the duration of the period in which norovirus was not detected (duration of interruption) and the number of samples is the number of samples that tested negative for norovirus before once again being detected.

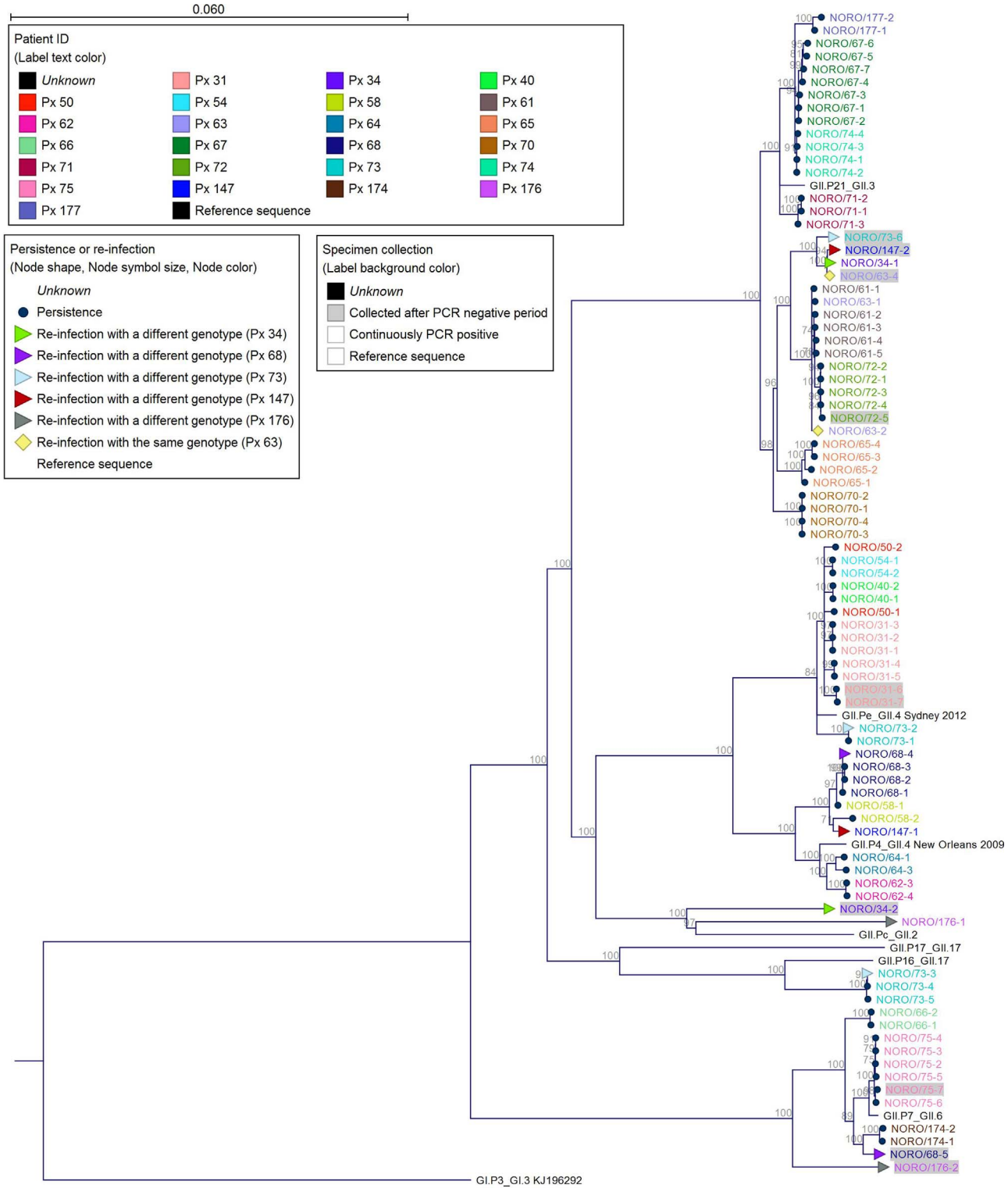

Fig. 1. Full genome maximum likelihood phylogeny of longitudinal norovirus sequences. Sequences are labelled with a unique patient number (Px, NORO/XX) and serial longitudinal numbering (e.g. NORO/XX-1). The node shape and colour indicates whether the position on the tree suggests persistence of the same virus, re-infection with a different genotype or re-infection with a different strain of the same genotype. Co-infections with multiple genotypes (Patient 65 and 101) are not shown since reliable consensus sequences for phylogenetic analysis cannot be generated.

Footnote: Longitudinal samples from Patients 58 and 63 (63-1 and 63-2) do not cluster as closely together as longitudinal samples from other patients; 445 and 135 days had passed between the longitudinal samples therefore is consistent with accumulation of mutations over time. The second sample from patient 58 clusters closely with samples from patient 68 and the early samples from Patient 63 cluster with samples from Patient 61; these patients were epidemiologically linked (data not shown).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jcv.2017.09.009.

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