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Impact of long-term storage of clinical samples collected from 1996 to 2017 on RT-PCR detection of norovirus

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Abstract

Noroviruses are recognized as the leading cause of acute gastroenteritis globally. With improved molecular diagnostics developed over the last two decades, archived clinical specimens are increasingly used to investigate the historic prevalence and molecular epidemiology of human norovirus. Yet the impact of long-term storage on viral integrity in clinical specimens has not been evaluated. In this study, we retested 994 stool specimens collected between 1996 and 2017 that originally tested norovirus-positive to quantify the loss of norovirus RT-PCR positivity with increasing sample storage time at 4 °C. In all, 79% of samples tested positive after retesting, but there was an approximate 3% decline in the positivity ratio and 4% decline in the percentage of samples that could be genotyped with each additional year of sample storage. For samples that were originally quantified by real-time RT-PCR (collected between 2003 and 2017), there was an estimated 1-log loss of viral titer occurring every 7 years of sample storage. Few samples contained PCR inhibitors, assessed using a MS2 extraction control, indicating that loss of RT-PCR signal was due primarily to loss of viral RNA integrity after long-term storage of stool samples at 4 °C. Our results indicate that norovirus positive stool samples can be stored with minimal loss in RT-PCR positivity when stored less than a decade. Longer periods of storage may impair norovirus detection, potentially impacting historic estimates of norovirus prevalence and molecular epidemiology if derived by testing archival clinical specimens.

Keywords

Norovirus; RT-PCR; Stool; Storage; Archival; Clinical; Surveillance

1. Introduction

Improved detection methods for noroviruses, in particular since the introduction of real-time RT-PCR in 2003, have been critical to demonstrate that noroviruses are the leading cause

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of acute gastroenteritis in all age groups and the primary cause of pediatric gastroenteritis in countries where rotavirus vaccination has been successfully implemented (Ahmed et al., 2014; Lopman et al., 2016; Payne et al., 2013). Symptoms of vomiting and/or diarrhea are normally self-limiting but can lead to hospitalizations and in rare cases deaths especially among children in developing countries and elderly adults in developed countries (Hall et al., 2013). Noroviruses are classified into at least 7 genogroups and further divided into at least 32 genotypes (Vinje, 2015). The majority of human infections are caused by genogroup (G) I and II viruses and in particular variants of the GII.4 genotype which emerge every 2–3 years resulting in epidemics and sometimes global pandemics since the mid-1990s (Green, 2013).

Noroviruses are shed in stools and emesis of infected persons and clinical diagnosis involves extraction of viral RNA from clinical samples and molecular detection. TaqMan-based real-time RT-PCR is the standard for clinical diagnosis and positive samples are genotyped by sequencing small regions of the major capsid protein-encoding region (ORF2; regions C and D) (Kroneman et al., 2013; Vega et al., 2014) or more recently by dual typing of both ORF1 and ORF2 (Cannon et al., 2017). Partial regions of the RNA polymerase encoding region have historically been used for norovirus typing but are less commonly used today (Anderson et al., 2001; Vinje and Koopmans, 1996). Clinical specimens are typically stored for short periods of time (days to weeks) prior to viral RNA extraction and detection, but studies involving archived clinical specimens have also shown that viral RNA can be detected after long-term storage of up to 40+ years (Amar et al., 2007; Bok et al., 2009; Boon et al., 2011; Koo et al., 2012; Mori et al., 2017; Rackoff et al., 2013; Siqueira et al., 2017). Like other enteric viruses transmitted by food-and water-borne routes, noroviruses can survive for long periods of time outside of the human host, particularly when protected by an organic material such as feces and when stored at refrigerated or frozen temperatures (Kotwal and Cannon, 2014). However, no laboratory studies have empirically assessed the RT-PCR positivity of viral RNA in clinical specimens stored for periods of time greater than one year.

The purpose of this study was to investigate the RT-PCR positivity of noroviruses in clinical specimens (stool) containing norovirus stored at 4 °C over a time period up to 22 years. Clinical samples (n = 994) collected between 1996 and 2017 and originally testing positive for norovirus by conventional and/or real-time RT-PCR were retested by real-time RT-PCR to determine positivity after sample storage. An internal extraction control (coliphage MS2) was included to assess RT-PCR inhibition of molecular methods and positive samples were genotyped after amplification and sequencing using a dual (polymerase and capsid) typing method (Cannon et al., 2017). Results are important for clinical diagnosis, particularly for projects investigating norovirus prevalence and molecular epidemiology of archival clinical specimens.

2. Materials and methods

2.1. Archived clinical specimen selection

Stool specimens (n = 994) collected between 1996 and 2017 and previously testing positive for GI and/or GII norovirus by conventional or real-time RT-PCR were stored at 4 °C since

the time of collection as part of routine outbreak surveillance conducted at the Centers for Disease Control and Prevention (CDC, Atlanta, GA). CDC's Internal Program for Research Determination deemed that this study is categorized as public health non-research and that human subject regulations did not apply. Conventional RT-PCR results were considered "positive" for norovirus if amplification by assays (targeting B, C or D regions) (Anderson et al., 2001; Blanton et al., 2006; Fankhauser et al., 2002; Vega et al., 2014; Vega et al., 2011; Zheng et al., 2006) produced PCR products of the appropriate size following gel electrophoresis; some but not all of these samples were originally genotyped by sequencing. Real-time RT-PCR results were considered "positive" when fluorescence resulting in a S-shaped curve was detected within 40 cycles of amplification by TaqMan assays (Trujillo et al., 2006; Vega et al., 2011; Vega et al., 2014). Semi-quantitative real-time results were reported when cycle threshold (Ct) values were within the assay limit of detection (LOD) using cut-off values for quantification (35 for GI and 37 for GII viruses) (Cannon et al., 2017). In this study, we randomly selected samples from each collection year (1996–2017) among those originally generating positive RT-PCR results as reported (Vega et al., 2011). At least two samples were included from each collection year, with a median of 44.5 (IQR 31.3–59.0) samples and maximum of 105 samples tested from each year (Table 1).

2.2. Methods of viral RNA extraction and real-time RT-PCR used during retest

Viral nucleic acid was extracted from 10% clarified fecal suspensions prepared in phosphate buffered saline using MagMax-96 Viral RNA Isolation Kit (Ambion, Foster City, CA, USA) according to the manufacturer's instructions on an automated KingFisher extractor (Thermo Fisher Scientific, Pittsburgh, PA, USA). All specimens were freshly extracted prior to retest as part of this study. Coliphage MS2 virus was added to each sample prior to RNA extraction serving as a control to assess possible viral loss during RNA extraction and inhibition of RT-PCR (Rolfe et al., 2007). Viral RNA was detected using a multiplex real-time RT-PCR assay (Cannon et al., 2017; Park et al., 2017; Vinje, 2015) with the Ag-Path Kit (Applied Biosystems, Carlsbad, CA, USA) and cycling conditions as described previously (Cannon et al., 2017).

2.3. Genotyping clinical specimens testing positive upon retest

RT-PCR (Polymerase-Capsid [P-C] dual typing assay) was performed as previously described (Cannon et al., 2017). PCR products 579 bp GI, 570 GII) were visualized on a 2% agarose gel (Seakem-ME, Lonza, Allendale, NJ, USA) containing Gel Red (Biotium, Fremont, CA, USA) and post-PCR product purified by ExoSAP-IT (Affymetrix, USB, Cleveland, OH, USA) or QIAquick Gel extraction kit (Qiagen) and sequenced by Sanger technology (Eurofins MWG Operon, Louisville, KY, USA). Genotypes were assigned by UPGMA phylogenetic analysis using reference sequences used by CaliciNet (Vega et al., 2011) for polymerase and capsid typing (Cannon et al., 2017; Vinje, 2015).

2.4. Analysis of data and statistical comparisons

Previous RT-PCR results and sample collection dates and retest results were extracted from our internal Viral Gastroenteritis database hosted by BioNumerics 7.6.3 (Applied Maths, Austin, TX, USA) which includes CaliciNet (Cannon et al., 2017) and imported into MS Excel (2016) for basic data manipulation. The ratio of samples testing positive after retest

over the number of samples originally testing positive and the ratio of samples that could be genotyped after retest over the total number of samples originally testing positive were calculated for each year of sample collection. Change in Ct value (Ct value of GI and GII samples after retest minus the originally determined Ct value of each samples) was calculated for all samples that were originally quantified by real-time RT-PCR. Change in MS2 extraction control Ct value (Ct value of MS2 included in each sample over the Ct value generated for MS2 RNA without sample) was used to screen for inhibition of RT-PCR.

JMP Pro 13.0.0 (SAS, Cary, NC, USA) was used for graphing and statistical analysis. Least square means regressions and Pearson correlations were plotted for positivity ratio, genotyping ratio, and change in Ct value estimates by year of sample collection and bivariate normal density ellipses (90% confidence) were plotted for individual GI, GII and originally "unknown" genotype data. Analysis of variance was used to identify significant differences ($p > 0.05$) between ratios of positivity and change in Ct values over time for GI and GII genogroups using the interaction variable to determine differences between slopes of the linear regressions. Significant differences in aggregate GI and GII Ct values generated originally were determined by a Mann–Whitney test ($p > 0.05$).

3. Results

3.1. Impact of sample storage time on GI and GII norovirus detection by RT-PCR

Of the selected 994 norovirus-positive archival clinical samples collected between 1996 and 2017, 780 (78.5%) tested positive upon retesting. Overall, the ratio of samples testing positive after retest was greater for samples that were collected more recently (Fig. 1). There was a general positive linear trend ($y = 0.028x–54.94$; $R^2 = 0.76$; Pearson's $r = 0.873$), with an approximate 2.7% (95% CI, 2.1–3.5%) decrease in the percent of samples testing positive with each additional year of sample storage (Fig. 1).

For samples that tested originally positive for GII viruses, percent positivity after retest ranged from 55% to 100% between the collection years of 2003–2017, with a median of 90.0% (IQR 83.3–98.5%). There was an approximate 2.1% (95% CI, 1.1–3.1%) decrease in positivity ($y = 0.021x–41.64$; $R^2 = 0.551$; Pearson's $r = 0.743$) for each year GII samples were stored (Fig. 2). For samples originally positive for GI viruses, percent positivity ranged from 33.3% to 100% between the collection years of 2002–2017, with a median of 80.0% (IQR 65.6–97.5%). The decline in positivity with increasing sample storage time did not differ for GI and GII samples (interaction variable $p = 0.776$) nor did the mean ratio of positivity ($p = 0.099$). However, linearity was not as strong for GI samples ($y = 0.017x − 33.7$; $R^2 = 0.123$; Pearson's $r = 0.351$) (Fig. 2). Originally, 290 (29.2%) samples tested positive for an "unknown" genogroup (Table 2) since they were originally tested using a duplex conventional assay that was used exclusively in this data set between the years of 1996 and 2001 and used in conjunction with other RT-PCR assays from 2002 to 2006. Of these samples, 14.8% were determined to be GI, 40.0% were GII, 0.7% were GI/GII, and 44.5% were negative after retest (Table 2). A positive linear trend was not observed for originally "unknown" genogroup samples by sample collection date (Pearson correlation = −0.064) and the median percentage of positive samples was 50.8% (IQR 50.0–58.0%) for this group (Fig. 2).

3.2. Impact of archival sample storage on dual typing

Originally, 394 (39.6%) of the 994 positive samples were genotyped. Of these, 77 (19.5%) were GI, 312 were GII (79.2%) and 5 (1.3%) were GI/GII. After retest, 297 (75.4%) could be genotyped. Of these, 53 (17.8%) were GI, 242 (81.5%) were GII, and 2 (0.7%) were GI/GII. Of the 297 genotyped retest samples, 91.2% (271) were typed as the same genotype originally determined. Among those that typed differently after retest, 5 were previously untypeable and 4 were mixed GI/GII infections for which one genotype was not detected originally. The remaining 17 (5.7%) of samples were typed as a different genotype upon retest. An additional 189 samples, with collection dates ranging from 1997 to 2017, could be genotyped after retest but were not originally genotyped. The distribution of genotypes was GI (40; 21.2%), GII (148; 78.3%), and GI/GII (1; 0.5%). Considering all 486 samples genotyped upon retest, there was a linear trend toward decreasing likelihood of genotyping success with increasing sample storage time ($y = 0.043x - 86.46$; $R^2 = 0.858$; Pearson's $r = 0.926$) (Fig. 3). With each year of sample storage there was an estimated 4.3% (95% CI, 3.6–5.1%) decline in genotyping success. Table 2 shows P-C genotyping results for retest samples by year of sample collection. There were no additional genotypes detected originally that were not detected with retest.

3.3. Quantification (CT value) of losses in RT-PCR signal with increased sample storage time

Semi-quantitative (Ct value) real-time RT-PCR results were originally available for 60.0% (596/994) of archival stool samples; 29.9% (297/994) were tested originally only by conventional RT-PCR and 10.2% (101/994) of samples were originally tested positive by real-time RT-PCR but Ct values were either not reported (82 samples) or Ct values were greater than cutoff values (35 for GI and 37 for GII) for quantification (19 samples). Upon retesting, 774 samples (77.9%) of the 994 samples were positive by real-time RT-PCR. Among the remaining samples, 22.1% (220/994) tested negative by real-time RT-PCR after retesting by not yielding a Ct value or yielding a Ct value that was beyond the cutoff limits for GI and GII. Ct values obtained originally were higher for GI versus GII virus containing samples ($p = 0.0014$) and a higher proportion (30.6%) of GI samples that gave a Ct value beyond the LOD after retesting compared to GII (11.0%) (Table 3).

For the samples which tested positive by real-time RT-PCR originally, the change in Ct value (Ct value after retest minus the original Ct value) was calculated for each sample and graphed by sample collection year (Fig. 4). Archival clinical specimens with earlier collection dates yielded larger changes in Ct values after retest than did samples that were retested near the time of sample collection (Fig. 4). This trend was observed for both GI and GII samples, as the slope of the regression lines [GI ($y = -0.826x + 1666$, $R^2 = 0.27$; Pearson's $r = -0.517$); GII ($y = -0.406x + 818.6$, $R^2 = 0.05$); Pearson's $r = -0.222$] were significantly different from zero (both $p < 0.0001$). This indicates there was a greater loss of RT-PCR signal (lower viral load detected) after retesting of older samples than those collected more recently. Yearly increases in Ct value (indicating a loss of viral RNA titer detected) were approximately 0.83 (95% CI, 0.49–1.16) and 0.41 (95% CI, 0.25–0.56) for GI and GII, respectively, and 0.46 (95% CI, 0.32–0.60) overall (for GI and GII data combined; $y = -0.462x + 931.4$; $R^2 = 0.065$; Pearson's $r = -0.256$). Rates of change

(slope of the linear regressions) over the sample collection years significantly differed for GI and GII samples ($p = 0.0264$) (Fig. 3). If an increase in Ct value of 3.3 is considered an approximate 1-log loss in viral RNA titer, overall there was a 1-log loss in viral RNA titer approximately every 7.1 (95% CI, 5.5–10.3) years of sample storage (4.0 years for GI samples and 8.1 years for GII samples).

3.4. Distinguishing between viral RNA loss during extraction or RT-PCR inhibition and loss of viral RNA integrity resulting in an RT-PCR negative result

Plotting changes in Ct values for each GI and GII sample against the change in Ct values generated for MS2 extraction controls allows visualization of loss in viral RNA integrity resulting in a RT-PCR negative result versus inhibition of RT-PCR or loss of viral RNA during extraction. Graphically, a shift to the right indicates a loss of GI or GII RT-PCR signal as the retest Ct value was higher than the original Ct value for that sample. It is expected that the Ct values obtained for MS2 included in each sample would be equivalent to the Ct values for the control MS2 sample plated with each RT-PCR run. However, a positive change in MS2 Ct value (shift upwards) is indicative of loss in MS2 RNA during extraction or RT-PCR inhibition. Graphically, if a positive change in Ct value for GI or GII is accompanied by a positive change in Ct value for MS2 for an individual sample, it would be indicative of either RT-PCR inhibition or inefficient viral RNA extraction. However, if the increased change in Ct value for GI or GII was not accompanied by an increased change in MS2 value, the loss in signal was more likely due to loss in viral RNA integrity. For this analysis we chose an arbitrary change in Ct value of 3 as a cutoff, as this is an approximate 1-log loss in RT-PCR signal. In all, there were 211 samples (49 GI and 162 GII) that had GI or GII change in Ct values > 3. Of these, only 7 (1 GI and 6 GII) were accompanied by change in MS2 Ct values > 3. Therefore, the remaining samples (204/211; 96.7%) showed evidence of loss in viral RNA integrity (Fig. 5).

In some cases, particularly for GII samples, Ct values after retest were lower than those generated originally (Fig. 5). A shift to the left accompanied by a shift downward (negative change in MS2 Ct value) would indicate a greater efficiency in sample extraction. However, only 1 GI and 3 GII samples generated both negative (< −3) change in Ct values for GI or GII and MS2, ruling out more efficient extraction for the majority of samples. Since original real-time RT-PCR testing was either performed at CDC or by state or local public health labs submitting to CDC, data was also examined by original testing location (internal or external), but no significant trends were observed for the location of original testing variable (data not shown). It is possible that some of the variability of results, particularly for older samples, is due to assay improvements that enhance detection sensitivity.

4. Discussion

We evaluated the impact of long-term (over 22 years) storage of stool samples at refrigerated conditions (4 °C) on norovirus RNA RT-PCR positivity. Results show a significant quantifiable loss of norovirus RT-PCR signal with increasing sample storage time. There was an approximate 3% (95% CI, 2–4%) decline in RT-PCR positivity and a 4% (95% CI, 4–5%) decline in the ability to genotype noroviruses in clinical samples with each additional

year of sample storage. If a change in Ct value of 3.3 is considered a 1-log loss in viral RNA titer, for each year of clinical sample storage, an estimated 1-log loss in viral RNA titer was detected with each 7 (95% CI, 6–10) year period of sample storage.

There are very few studies investigating the effect of duration of sample storage on molecular detection of microorganisms, particularly for viruses, in clinical samples. Most published studies pertain to microbiome research since there has been some noted difficulties in study reproducibility, prompting a thorough investigation of quality control parameters (including sample storage) impacting study outcomes (Sinha et al., 2015). A recent review found generally that sample storage for hours under refrigerated (4 °C) conditions or days to months under freezing (−20 °C or −80 °C) conditions did not impact bacterial community structure or species richness (Klymiuk et al., 2016), but there was a paucity of data for clinical samples (in the absence of preservation media) stored longer than one year. Studies on virus survival pertain mostly to water, food and environmental matrices, but scarcely include time periods greater than one year (Kotwal and Cannon, 2014). However, one study found that norovirus RNA could be detected in seeded groundwater after 3.5 years of refrigerated storage with only a 1-log reduction in RT-PCR signal (Seitz et al., 2011). Fecal material has a protective effect on virus survival (Kotwal and Cannon, 2014), which is perhaps the reason similar reductions in RT-PCR signal were detected only after seven years in the current study. While several studies have investigated the prevalence and molecular epidemiology of noroviruses in archived stool specimens collected 20+ years ago (Amar et al., 2007; Bok et al., 2009; Boon et al., 2011; Mori et al., 2017; Rackoff et al., 2013; Siqueira et al., 2017), the impact of duration of sample storage (at −20° C or −80° C) was not evaluated.

Loss of GI viral RNA integrity did appear to be greater than losses in GII integrity in this study, but differences in viral titers complicated this comparison. For samples that were quantified by real-time RT-PCR, Ct values for GI viruses were higher than those of GII viruses, indicating a lower virus titer for GI samples. However, this difference, which was more pronounced after retest, may be inflated since samples testing beyond the LOD were ascribed a Ct value of 40 to prevent excluding them from further analysis. More GI samples were beyond the LOD after retesting (30%) than GII samples (11%). It is possible that the greater loss of RT-PCR signal observed for GI viruses after retesting is due primarily to difference in initial titer rather than loss in integrity. There is mixed evidence in the literature when comparing the environmental stability of GI versus GII viruses which may also be influenced by type of environmental matrix (Matthews et al., 2012) and also stability differences between genotypes within each genogroup (Park et al., 2016).

Nearly all retest sample genotyping results (using the P-C dual typing assay) matched the originally reported genotypes (determined primarily using region C or D). There were some exceptions noted for mixed GI/GII outbreaks, where only one genotype was detected with retest, and where previously “untypeable” genotypes were reported but typing could be assigned with retest. A subset of retest samples were typed as a different genotype than originally determined. For these samples it is possible that mixed infections with more than one norovirus genotype were present in the stool samples. Since all the genotypes originally reported were also detected with retest, it seems that the dual typing assay was at least

as sensitive as previously used genotyping methods. Considering the diversity of different genotypes detected, it was not possible to determine an association between genotype and frequency of false-negative detection upon retest.

Analysis of MS2 extraction control Ct values showed that inhibition of RT-PCR or inefficient extraction of RNA was minimal in retest samples, supporting our findings that losses in GI and GII RT-PCR signal are due primarily to losses in viral RNA integrity. This finding is complicated by the nature of comparing semi-quantitative RT-PCR results. It is possible that the negative change in GII Ct values observed were due to assay improvements over the last 20 years, but it may also be indicative of variability inherent when there are differing conditions for RT-PCR data generation. While the GI and GII primers and GII probe sequences used currently for real-time detection remained unchanged since initial introduction of the assay, updates to the GI probe have been made and the assay is now multiplexed for simultaneous detection of GI and GII norovirus and an MS2 extraction control (Cannon et al., 2017; Kageyama et al., 2003; Trujillo et al., 2006; Vega et al., 2014; Vega et al., 2011). Although viral RNA extraction has remained based on lysis of virus particles using guanidinium isothiocyanate (Boom et al., 1990) throughout the study period, changes to the RNA extraction and master mix kits and real-time detection platforms used likely contribute to differences in Ct values that are not related to actual changes in viral RNA titer in the samples. The samples used in our study include those tested at the CDC as well as those submitted by public health labs. While harmonization of norovirus detection methods used by US state and local labs began with the launch of CaliciNet in 2009, differences in thermocycling platforms and RT-PCR kits cannot be ruled out to explain some of the observed differences. Ideally, including a standard curve in each assay for true quantification would be the best approach to quantify losses in viral RNA integrity.

Samples collected in 1996–2002 had primarily been tested with a region B conventional RT-PCR assay (Anderson 2001). Interestingly, roughly only half of these samples were positive after retest. Since not all amplicons were confirmed by DNA sequencing, it is possible that some of these PCR products were false positive and true losses in viral RNA integrity for those samples cannot be determined. With the start of CaliciNet in 2009, norovirus outbreaks submitted must be accompanied by DNA sequence confirmation for at least two RT-PCR positive samples, ruling out submission of outbreaks with false-positive results.

This study is the first to quantify loss of viral RNA RT-PCR positivity with prolonged storage of clinical samples at refrigeration temperature. Results indicate that stool samples can be stored for approximately 7 years before there are significant losses in norovirus detection. Clinical specimens in this study were stored at 4 °C, but future studies should also assess the impact of long-term storage at freezing temperatures (−20 °C and −80 °C) and the impact of cycles of freezing and thawing as samples collected as a part of the WHO global network for rotavirus surveillance have been stored at −20 °C (WHO, 2008). This study has implications for historic estimates of norovirus prevalence and molecular epidemiology if derived by testing archival clinical specimens.

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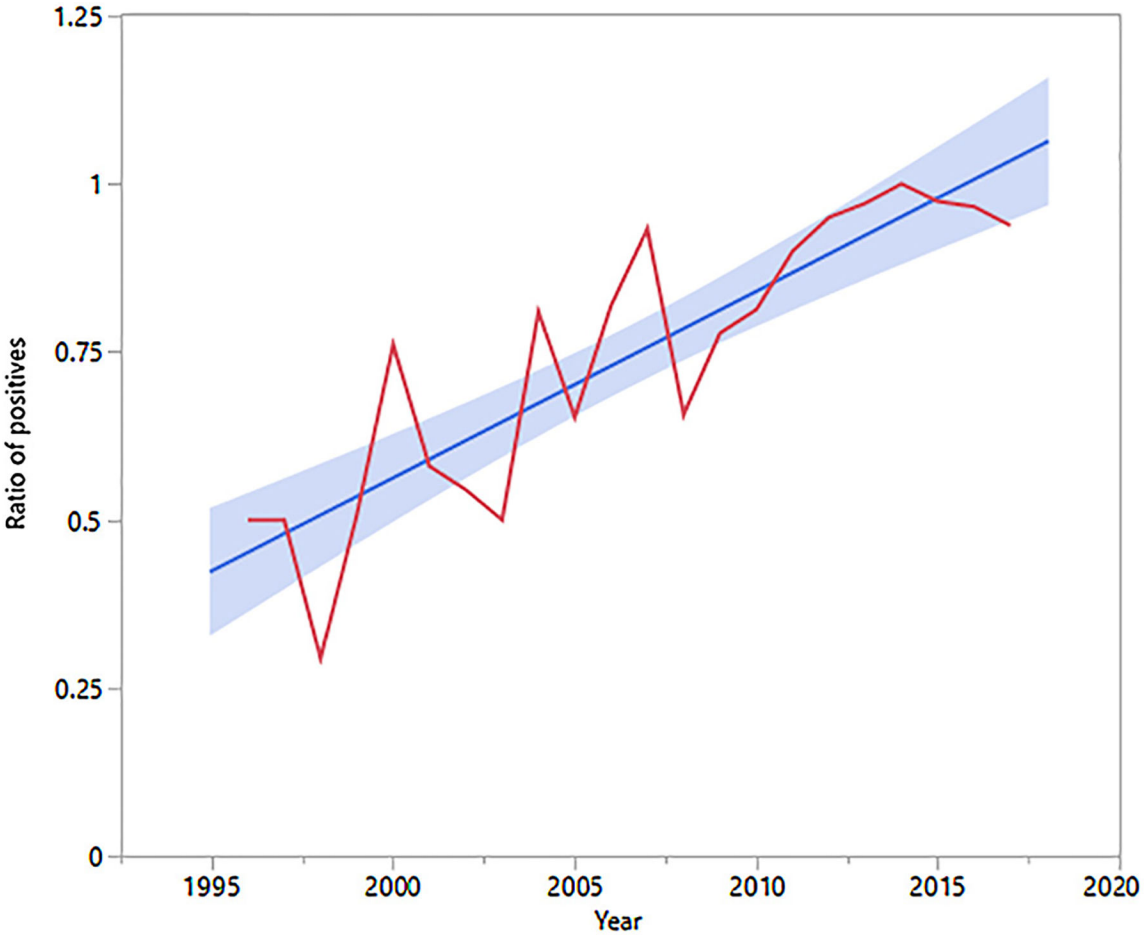

Fig. 1.

Ratio of archival stool samples testing positive for norovirus after retest, by year of sample collection. Red line connects individual data points. The linear fit with 95% confidence band is shown in blue.

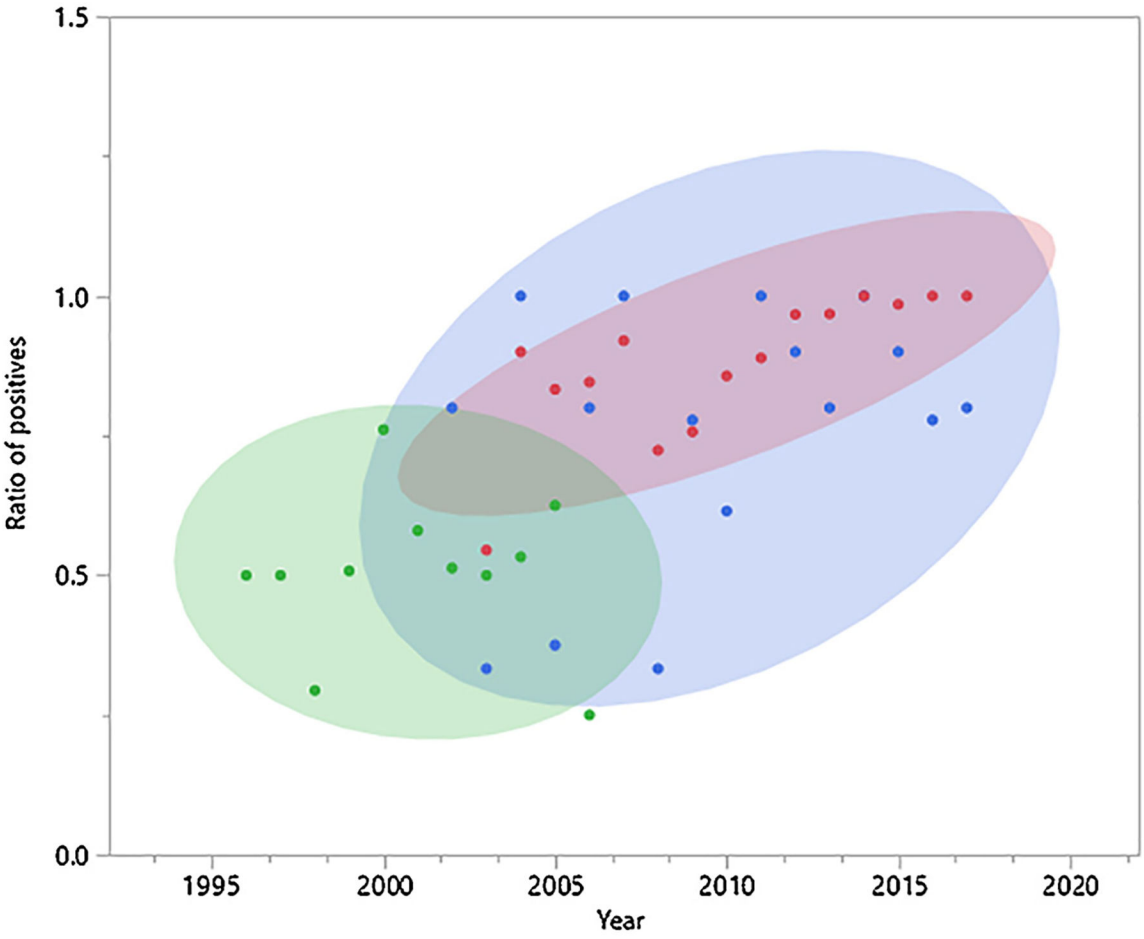

Fig. 2.

Ratio of archival stool specimens testing positive after retest by year of sample collection, grouped by originally determined genogroup. Bivariate normal density ellipses with 90% coverage are shown for samples originally typed as “unknown” (green), GI (blue), and GII (red).

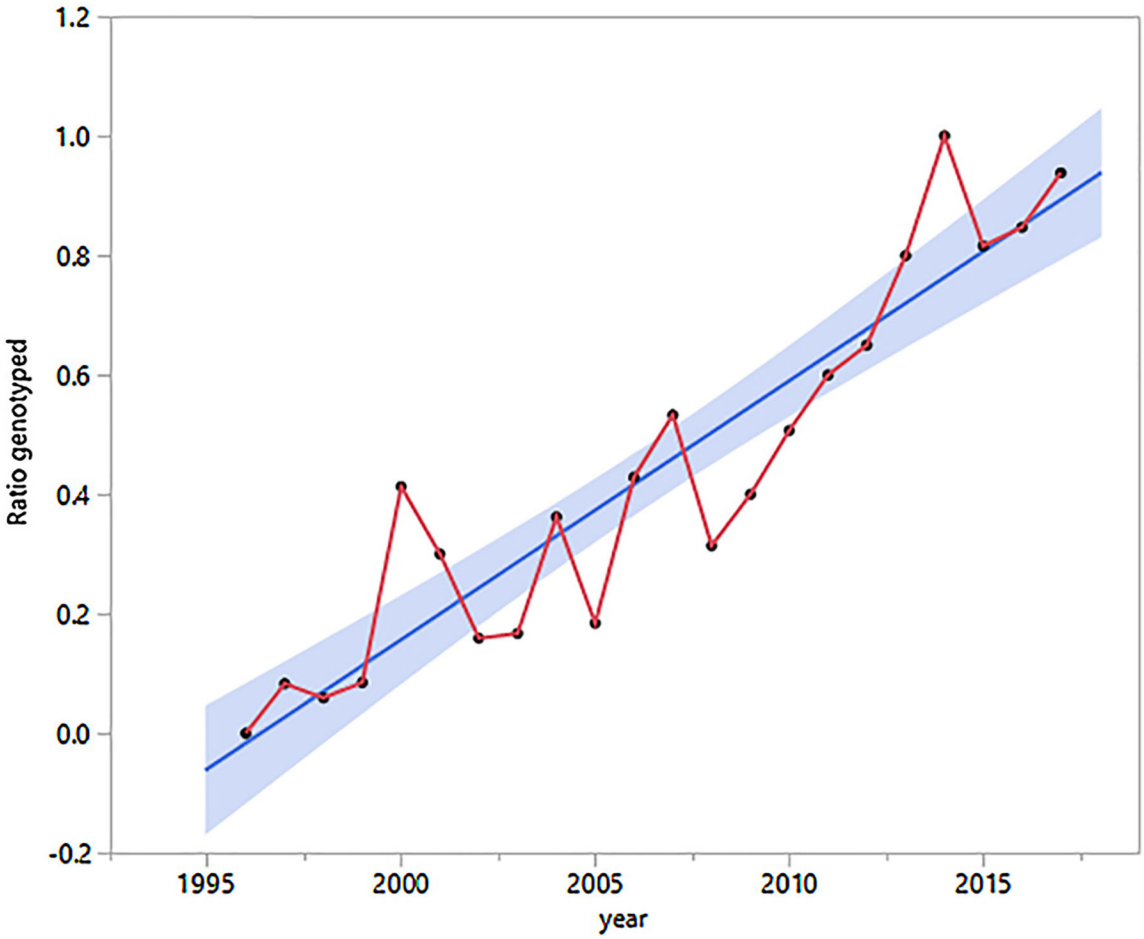

Fig. 3.

Ratio of archival norovirus positive stool samples that could be genotyped after retest, by year of sample collection. Red line connects individual data points. The linear fit with 95% confidence band is shown in blue.

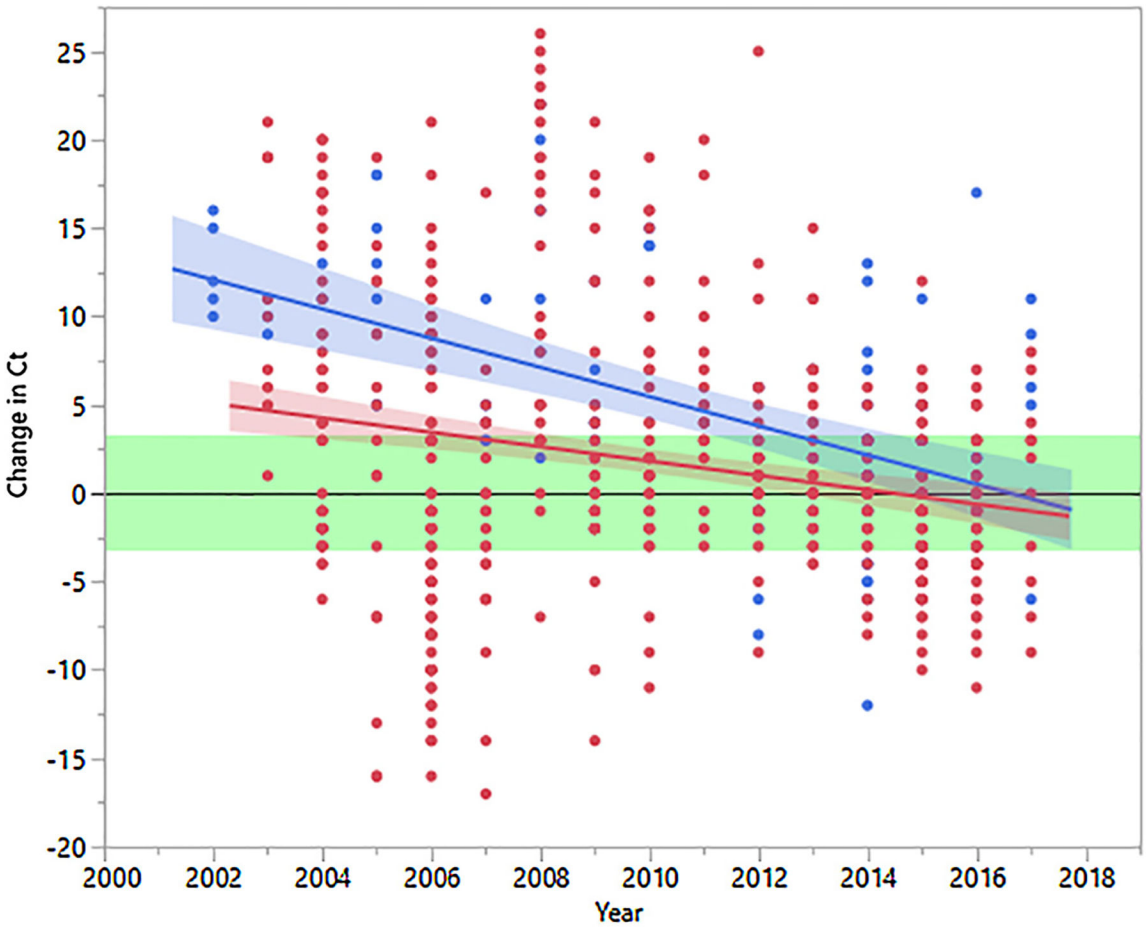

Fig. 4.

Change in Ct value calculated after retest for all GI and GII norovirus positive archival stool samples which could be originally quantified, by year of sample collection. Linear regressions with 95% confidence bands are indicated for GI (blue) and GII (red) samples. No change in Ct is indicated with a solid black horizontal line with a green area approximating a 1-log change in Ct value (range −3 to 3).

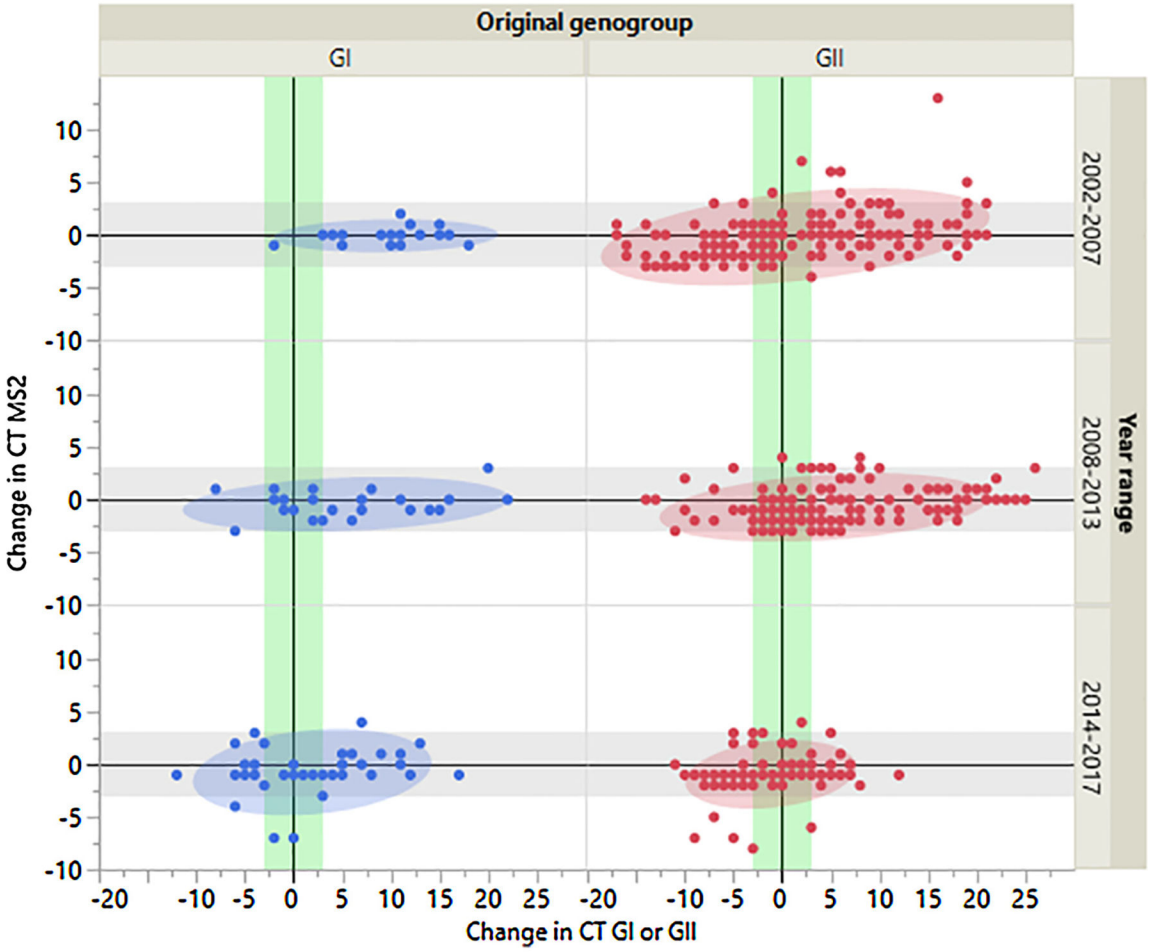

Fig. 5.

Change in GI and GII norovirus Ct values associated with sample storage time and inhibition of RT-PCR assessed using MS2 extraction control. Individual sample results and 90% bivariate normal density ellipses are shown for GI (blue) and GII (red) data. No change in Ct for GI and GII are indicated with a vertical black line with a green area approximating a 1-log change in Ct value (range −3 to 3). No change in Ct for MS2 is indicated with a horizontal black line with a gray area approximating a 1-log change in Ct value (range −3 to 3).

Table 1

Number of norovirus positive archival stool specimens samples (collected from 1996–2017) selected for retesting in 2017, by year of sample collection and genogroup.

Year	GI # (%)	GII # (%)	GI/GII # (%)	Unknown # (%)	Total #
1996	0 (0)	0 (0)	0 (0)	2 (1)	2
1997	0 (0)	0 (0)	0 (0)	12 (1)	12
1998	0 (0)	0 (0)	0 (0)	17 (1)	17
1999	0 (0)	0 (0)	0 (0)	59 (1)	59
2000	0 (0)	0 (0)	0 (0)	46 (1)	46
2001	0 (0)	0 (0)	0 (0)	50 (1)	50
2002	5 (0.11)	0 (0)	0 (0)	39 (0.89)	44
2003	3 (0.08)	11 (0.31)	0 (0)	22 (0.61)	36
2004	3 (0.05)	40 (0.69)	0 (0)	15 (0.26)	58
2005	7 (0.14)	17 (0.35)	1 (0.02)	24 (0.49)	49
2006	10 (0.1)	91 (0.87)	0 (0)	4 (0.04)	105
2007	5 (0.17)	25 (0.83)	0 (0)	0 (0)	30
2008	6 (0.17)	29 (0.83)	0 (0)	0 (0)	35
2009	8 (0.18)	36 (0.8)	1 (0.02)	0 (0)	45
2010	12 (0.16)	62 (0.83)	1 (0.01)	0 (0)	75
2011	3 (0.1)	27 (0.9)	0 (0)	0 (0)	30
2012	10 (0.25)	30 (0.75)	0 (0)	0 (0)	40
2013	4 (0.11)	30 (0.86)	1 (0.03)	0 (0)	35
2014	31 (0.41)	44 (0.59)	0 (0)	0 (0)	75
2015	10 (0.13)	66 (0.87)	0 (0)	0 (0)	76
2016	7 (0.12)	50 (0.85)	2 (0.03)	0 (0)	59
2017	5 (0.31)	11 (0.69)	0 (0)	0 (0)	16
All years	129 (0.13)	569 (0.57)	6 (0.01)	290 (0.29)	994

Table 2

Norovirus genotypes detected in archival samples upon retest, by year range of sample collection.

Year range	GI genotypes	GII genotypes
1997–2001	GI.P1-GI.1	GII.P2-GII.2
	GI.P3-GI.3	GII.P3-GII.3
	GI.Pd-GI.3	GII.P21-GII.3
	GI.P4-GI.4	GII.P4-GII.4 US95-96
	GI.P8-GI.8	GII.P4-GII.4 Henry
	GI.Pa-GI.3	GII.P5-GII.5
		GII.P6-GII.6
		GII.P7-GII.6
		GII.P7-GII.7
		GII.P12-GII.10
		GII.P16-GII.16
2002–2007	GI.P3-GI.3	GII.P2-GII.2
	GI.P4-GI.4	GII.P21-GII.3
	GI.Pb-GI.6	GII.P4-GII.4 Farmington Hills
		GII.P4-GII.4 Hunter
		GII.P4-GII.4 Yerseke
		GII.P4-GII.4 Den Haag
		GII.P4-GII.4 Sydney
		GII.P12-GII.4 Asia
		GII.P22-GII.5
		GII.P7-GII.7
2008–2013	GI.P3-GI.3	GII.Pg-GII.1
	GI.P4-GI.4	GII.P2-GII.2
	GI.P5-GI.5	GII.Pe-GII.4 Osaka
	GI.Pb-GI.6	GII.P4-GII.4 Apeldoorn
	GI.P7-GI.7	GII.P4-GII.4 Den Haag
		GII.P4-GII.4 New Orleans
		GII.P4-GII.4 Sydney
		GII.Pe-GII.4 Sydney
		GII.P22-GII.5
		GII.P7-GII.6
		GII.P7-GII.7
		GII.Pg-GII.12
		GII.P7-GII.14
		GII.P21-GII.21
		GII.P25-GII.25
2014–2017	GI.P2-GI.2	GII.P2-GII.2
	GI.P3-GI.3	GII.P16-GII.2
	GI.Pd-GI.3	GII.P21-GII.3

Year range	GI genotypes	GII genotypes
	GI.P5-GI.5	GII.P4-GII.4 Den Haag
	GI.Pb-GI.6	GII.P4-GII.4 Sydney
	GI.P7-GI.7	GII.Pe-GII.4 Sydney
		GII.P16-GII.4 Sydney
		GII.P7-GII.6
		GII.P16-GII.13
		GII.P17-GII.17

There were 5 additional samples with untypeable polymerase types and 2 with untypeable capsid genotypes

Table 3

Aggregate real-time RT-PCR Ct values for norovirus GI and GII samples.[a]

	GI (range)	GII (range)
Median original Ct (IQR)	25 (22–28)	23 (19–26)
Median retest Ct (IQR)	29 (23–38)	23 (19–29)

[a]The percentage of samples giving Ct values beyond the limit of detection for GI and GII viruses after retest were 30.6% (30/98) samples, and 11% (55/502) samples, respectively.