Multicenter evaluation of the revised RIDA® QUICK test (N1402) for rapid detection of norovirus in a diagnostic laboratory setting

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ABSTRACT

The updated RIDA® QUICK (N1402) immunochromatographic assay (R-Biopharm) for detection of norovirus was evaluated during a prospective, multicenter study using 771 stool samples from patients with gastroenteritis. Compared to real-time reverse transcriptase polymerase chain reaction (RT-rtPCR) as gold standard, the RIDA® QUICK had an overall sensitivity of 72.8% (91/125) and a specificity of 99.5% (640/643). Genotype analysis of the polymerase (ORF1) and capsid (ORF2) region of the genome indicated that the RIDA® QUICK assay could detect a broad range of genotypes including new variants (15 of 125 positive samples) which were detected by an in-house SYBR®Green RT-rtPCR, but not by the RIDA® GENE PCR PG1415 (R-Biopharm) and mostly not by the RIDA® GENE PCR PG1405 and the Xpert® Norovirus assay (Cepheid). The RIDA® QUICK can be used to reliably confirm norovirus in stool samples, but a negative result does not definitively exclude the presence of norovirus.

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1. Introduction

Noroviruses are the leading cause of nonbacterial acute gastroenteritis in both children and adults (Patel et al., 2009). The CDC estimates that more than 21 million cases of acute gastroenteritis occur each year in the United States due to norovirus infection (CDC, 2010). Noroviruses are small, non-enveloped viruses with a single-stranded RNA. They can be classified into 7 genogroups (GI to GVII) based on amino acid sequence diversity in the major capsid protein of norovirus VP1 (Vinjé, 2015). Each genogroups is further divided into genotypes. So far, human pathogens have only been described from genogroup I (GI), genogroup II (GII) and genogroup IV (GIV) (Green, 2013). Noroviruses are highly contagious, since very low doses of viral particles can cause infection (Glass et al., 2009). They are excreted in stool and vomit. Transmission occurs by person-to-person spread and by ingestion of contaminated food or water. These characteristics facilitate rapid spreading of norovirus, causing both sporadic cases and outbreaks of gastroenteritis (Marshall and Bruggink, 2006). Rapid laboratory diagnosis therefore is essential to implement measures to prevent and control the outbreaks.

Real-time reverse transcriptase polymerase chain reaction (RT-rtPCR) is accepted as a gold standard for norovirus detection due to its high sensitivity and specificity. However, it is a technically demanding, laborious and expensive technique, limiting its usefulness in an outpatient setting or in a laboratory setting where molecular based techniques are not readily available. Therefore, antigen tests remain a useful tool for the rapid detection of norovirus in clinical practice. The RIDA® QUICK Norovirus (R-Biopharm AG, Darmstadt, Germany) is one of the most popular antigen tests and has already been evaluated in several studies (Ambert-Balay and Pothier, 2013; Battaglioli et al., 2012; Bruggink et al., 2011; Bruins et al., 2010; Derrington et al., 2009; Geginat et al., 2012; Kirby et al., 2010; Pombubpa and Kittigul, 2012). Although their specificity is generally satisfying, the RIDA® QUICK demonstrates lower sensitivities compared to RT-rtPCR, ranging from 52% to 83%. Consequently, the RIDA® QUICK Norovirus was revised and additional antibodies were included for a broad range of detection (article number N1402 instead of the former N1403). Also, the antibodies are dissolved in the reagent, instead of dried on the membrane, converting the test from a two-step flow through immunochromatographic test to a one-step lateral flow assay, thereby increasing the ease-of-use (Bruggink et al., 2015). To our knowledge, there is only one publication evaluating the RIDA® QUICK N1402 assay which retrospectively tested 100 RT-rtPCR positive (before and after freezing/thawing) and 112 RT-rtPCR negative fecal samples (Bruggink et al., 2015). The authors

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reported a sensitivity of 83% (prior to freezing/thawing) to 87% (after freezing/thawing) and a specificity of 97%. We present a prospective, multicenter study evaluating the performance of the RIDA® QUICK Norovirus N1402 on stool samples using RT-rtPCR as gold standard in a diagnostic laboratory setting.

2. Materials and methods

2.1. Ethics

Approval was obtained from the Ethics Committee form the principal study site (OLV Hospital Aalst) under Belgium registration number B126201630222. Nonetheless, as this study is carried out on left-over samples that were not taken for study purposes, it can be regarded as a retrospective, non-interventional study that does not apply to the Belgian law of May 7th 2014, according to circular number 455 and 472 (http://www.fagg-afmps.be/nl/items/lijst_omzendbrieven). Therefore, there is no essential need for approval by the Ethics committee.

2.2. Participant enrollment and sample collection

This prospective, multicenter clinical trial was conducted from November 2014 through April 2015 at five non-university hospital study sites in Belgium: HH Hospital in Lier (521 beds), Imelda Hospital in Bonheiden (502 beds), Oost-Limburg Hospital in Genk (811 beds), Jan. Yperman Hospital in Ieper (530 beds) and OLV Hospital in Aalst (844 beds). The participating study sites included both hospitalized and ambulatory patients from which a stool sample was sent to the laboratory for routine diagnostics of diarrheagenic pathogens. Only liquid or loose stool samples that were non-bloody after visual inspection were included. Only remnant specimens were used for testing with the RIDA® QUICK and one sample per subject was included in the study. Specimens were anonymized upon enrollment. A total of 779 patients were included across all study sites. Enrollment statistics by site are presented in Table 1. If the specimen was not tested immediately, it was stored at 2–8 °C until analysis for a maximum of 7 days (median: 1 day, 2.5–97.5 percentile: 0–7 days). The RIDA® QUICK was performed at each site, while the RT-rtPCR was carried out at the principal site. Samples for PCR were stored at −80 °C until RT-rtPCR was performed.

2.3. RIDA® QUICK Norovirus (N1402, R-Biopharm AG)

The test was performed according to manufacturer's instructions using 50 μL or 50 μg stool (Package insert RIDA® QUICK Norovirus, 2012). This rapid test is a one-step lateral flow immunochromatographic assay employing both biotinylated and gold-labeled anti-norovirus antibodies. When noroviruses are present in the positive specimen, immune complexes form with the gold-labeled anti-norovirus antibodies and migrate through the reaction membrane. Streptavidin captures the migrating immune complexes at the test line (T line) via the biotin coupled to the anti-norovirus antibodies, resulting in red-violet staining of the T line. Migrating gold-labeled antibodies not bound in the complex are bound later at the control line (C line). If norovirus antigens are not present in the specimen, the binding of gold-labeled immunocomplexes will not occur at the T line but only at the C line. The presence of a red C line confirms that the test was valid. The result was read visually after 15 minutes of incubation by a laboratory technician who was blinded for other test results. A norovirus positive control (NP1404, R-Biopharm AG, not included in the kit) was performed once every package. Specimen testing only proceeded in the event of a valid quality control result.

2.4. RIDA® GENE Norovirus I and II (PG1415, R-Biopharm AG)

First-line PCR testing was performed on all study samples using the RIDA® GENE Norovirus I and II RT-rtPCR (article number PG1415, R-Biopharm AG, Darmstadt, Germany), which is a multiplex RT-rtPCR for the direct, qualitative detection and differentiation of norovirus GI and GII, targeting the ORF1/ORF2 junction region. According to manufacturer's validation, the detection limit for norovirus is 50 RNA copies per reaction (10 RNA copies per μL) both for norovirus GI and GII (Package insert RIDA®GENE Norovirus I and II, 2014). Stool samples were diluted 1/10 with DNase and RNase free water and vortexed intensely. The suspension was centrifuged for 1 minute at 12000 rpm. The supernatant was extracted on the NucliSens EasyMAG (bioMérieux, Marcy L'Etoile, France) using the generic protocol (200 μL input, 20 μL Internal Control RNA and 60 μL elution volume). One step RT-rtPCR was performed on the ABI 7500 Fast (Life Technologies, Salt Lake City, USA) according to manufacturer's instructions. The temperature profile used was: 10 minutes 58 °C; 1 minute 95 °C; 45 cycles composed of 15 seconds 95 °C and 30 seconds 55 °C. The RT-rtPCR had documented good External Quality Control results in the QCMD quality program during the study. In case of inhibition, the RT-rtPCR was repeated on a 1/10 diluted extract. When inhibition control failed again, RNA extraction was repeated. In case of inhibition using the second extract, the sample was excluded from the study.

2.5. Additional PCR testing

RIDA® QUICK positive samples that were not confirmed by the RIDA® GENE Norovirus I and II (PG1415) were sent to the National Reference Center (NRC) for additional PCR testing. The samples were tested by three RT-rtPCRs: (i) an in-house SYBR®Green RT-rtPCR using the primers described in ISO/TS15216:2012 targeting the ORF1-ORF2 region of the norovirus genome. The presence of a positive PCR signal and a melting peak between 71 and 72 °C indicated the presence of norovirus; (ii) the RIDA® GENE Norovirus detection kit PG1405 targeting genogroups GI, GII and GIV; and (iii) the Xpert® Norovirus assay (Cepheid, Sunnyvale, CA) which is a commercial RT-rtPCR (Gonzalez et al., 2016). Samples that were tested with the Xpert® Norovirus assay underwent an additional freeze–thaw cycle.

2.6. Genotyping

Strains were further characterized to genotype level by partial sequencing of the polymerase and capsid regions using JV12(fw)-JV13(rv) primers (Vennema et al., 2002) for the polymerase region and using primer sets G1SKF/G1SKR to amplify 330 bp of the capsid region of norovirus GI and G2SKF/G2SKR to amplify 344 bp of the capsid region of GII genogroups (Kageyama et al., 2003). The PCR amplification products were sequenced using the ABI BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) in an automated ABI 3500 L genetic analyzer (Applied Biosystems). The partial sequences

Table 1
Enrollment statistics for evaluable specimens for all study sites (S).

	S1	S2	S3	S4	S5	Total
Gender						
Male	43	54	39	130	83	349
Female	53	87	41	116	125	422
Age						
≤10 years	15	26	9	115	29	194
11–30 years	7	7	9	7	10	40
31–50 years	11	3	11	16	20	61
51–70 years	25	29	20	39	44	157
>70 years	38	76	31	69	105	319
Inpatient status						
Hospitalized	71	109	73	170	165	588
Ambulatory	25	32	7	76	43	183
Stool consistency						
Liquid	35	40	57	100	97	329
Loose	61	101	23	146	111	442
Total	96	141	80	246	208	771

S1: HH Hospital (Lier); S2: Imelda Hospital (Bonheiden); S3: Oost-Limburg Hospital (Genk); S4: Jan. Yperman Hospital (Ypres); S5: OLV Hospital (Aalst).

of the polymerase and/or the capsid genes were used for genotyping using the Norovirus Genotyping Tool Version 1.0 (Kroneman et al., 2011).

2.7. Statistical analyses

Performance characteristics, including sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the RIDA® QUICK was calculated and expressed as a 95% confidence interval (CI) using RT-rtPCR as gold standard. A sample was considered norovirus positive if at least one of the RT-rtPCR's was positive (RIDA® GENE Norovirus I and II (PG1415), RIDA® GENE Norovirus detection kit (PG1405), Xpert® Norovirus assay or in-house SYBR®Green RT-rtPCR). The kappa statistics (linear weights) were used to compare test agreement. A Student's t-test or one-way ANOVA was used to compare means and a Fischer's exact test was used to test the significance of contingency. Multiple logistic regression was used to explore the effect of covariates on the presence of norovirus. A P-value <0.05 was considered statistically significant. Statistical analysis was performed with Microsoft Office Excel 2003 software (Microsoft Corporation, Redmond, WA, USA), MedCalc Version 12.3.0.0 (MedCalc Software bvba, Ostend, Belgium) and SPSS Statistics 23 (SPSS, Chicago, USA).

3. Results

A total of 779 liquid or loose stool samples were included in the study of which 771 were eligible for study inclusion. Eight samples (1.0%) were excluded because repeated inhibition of the RT-rtPCR. The remaining 771 evaluable specimens are listed by gender, age group, inpatient status and stool consistency for all study sites in Table 1. Overall, 349 males (45.3%) and 422 (54.7%) females were included with a median age of 63 years (2.5th–97.5th percentile: 0–92 years). The overall positivity rate of norovirus was 16.2% (125/771) (norovirus GI: 0.6% (5/771) and GII: 15.6% (120/771)), although marked local difference was noticed (range: 7.3–26%). The RIDA® GENE that was used as first line RT-rtPCR test detected 110 positive samples. Another 15 were detected by the in-house SYBR®Green RT-rtPCR in the NRC when analyzing RIDA® GENE (PG1415) negative/RIDA® QUICK positive samples. Only three of those 15 were found positive with the RIDA® GENE Norovirus detection kit PG1405, and only two with the Xpert® Norovirus assay. Multivariate logistic regression showed that age was the only parameter that was independently correlated with a positive result for norovirus (b = −0.016, $P < 0.001$). However, age was not significantly related to the viral load of positive samples, expressed by the Ct-value of norovirus GII positive samples by the RIDA® GENE (r = 0.11, $P = 0.28$).

Sequencing analysis was performed on the partial polymerase gene and on the capsid gene. In 30 samples the norovirus strain could not be genotyped as the routine primers failed to amplify the norovirus RNA. Genotype GI.3 was confirmed in one sample based on the capsid sequence. In 57 of the GII positive samples the capsid gave a sequencing result with as dominant genotype GII.4 (37/57). Other genotypes were GII.1 (9/57), GII.2 (3/57), GII.3 (2/57), GII.6 (2/57), GII.7 (3/57) and GII.14 (1/57). Polymerase sequence resulted in 60 evaluable samples, with GII. P4 as dominant genotype (35/60) followed by GII. Pe (20/60). Other genotypes found were GII. P2 (2/60), GII. P7 (1/60), GII. P15 (1/60) and GII. P21 (1/60). The 15 RIDA® GENE negative/RIDA® QUICK positive samples were also analyzed only based on the polymerase region, as the capsid region gave no amplification product. GII. P4 was found in 8 samples, whereas GII. Pg was detected in one sample. In all the 5 study sites participating in this study, GII.4 was present during the sampling period. In hospital study site 4, a bigger variation in genotypes was noticed compared to the other participating hospitals. This variation was noticed for both polymerase and capsid genes (Table 2).

The performance characteristics of the RIDA® QUICK for detection of norovirus GI and GII compared to RT-rtPCR as gold standard are

Table 2

Different genotypes and GII.4 variants found during the study in the different study sites (S) based on the partial polymerase and capsid sequence.

Study site	Polymerase genotype (n° of samples)	Capsid genotype (n° of samples)
S1	GII. P4 (4)	GII.4 could not assigned (1)
		GII.4 Sydney 2012 (2)
S2	GII. P4 (1)	GII.1 (2)
	GII. P4 New Orleans (6)	GII.4 Sydney 2012 (11)
	GII. P4 could not assigned (1)	GII.6 (1)
	GII. Pe (5)	GII.14 (1)
S3	GII. P2 (1)	GII.2 (1)
	GII. P4 (1)	GII.4 Sydney 2012 (1)
	GII. P4 New Orleans (2)	
	GII. Pe (1)	
S4	GII. P2 (1)	GI.3 (1)
	GII. P4 (8)	GII.1 (6)
	GII. P4 New Orleans (6)	GII.2 (2)
	GII. P4 could not assigned (1)	GII.3 (1)
	GII. P7 (1)	GII.4 Sydney 2012 (19)
	GII. P15 (1)	GII.6 (1)
	GII. Pe (11)	GII.7 (3)
S5	GII. P4 (3)	GII.1 (1)
	GII. P4 could not assigned (2)	GII.3 (1)
	GII. P21 (1)	GII.4 (1)
	GII. Pe (3)	GII.4 Sydney 2012 (2)

S1: HH Hospital (Lier); S2: Imelda Hospital (Bonheiden); S3: Oost-Limburg Hospital (Genk); S4: Jan. Yperman Hospital (Ypres) S5: OLV Hospital (Aalst).

presented in Table 3. Overall sensitivity and specificity was 72.8% and 99.5%, respectively. However, high inter-laboratory variability in sensitivity was observed (ranging from 56.3% to 100%). False negative samples had a significantly higher Ct-value compared to true positive samples, both for norovirus GI ($P = 0.02$) and GII ($P < 0.001$, see Fig. 1). There was a significant difference in Ct-value between the different centers ($P = 0.006$).

4. Discussion

In this multicenter study, the RIDA® QUICK N1402 antigen test for the detection of norovirus GI and GII was evaluated on liquid and loose, non-bloody stool samples. Compared to RT-rtPCR as gold standard, the test exhibited a sensitivity of 72.8% and a specificity of 99.5%. These results are similar to those previously published for the RIDA® QUICK N1403 (Ambert-Balay and Pothier, 2013; Battaglioli et al., 2012; Bruggink et al., 2011; Bruins et al., 2010; Derrington et al., 2009; Geginat et al., 2012; Kirby et al., 2010; Pombubpa and Kittigul, 2012) and RIDA® QUICK N1402 (Bruggink et al., 2015), although direct comparison is not applicable for the RIDA® QUICK N1402 as this is the first prospective cohort study. In general, there is an inferior sensitivity of norovirus antigen tests compared to molecular assays. This seems to be strain dependent as a reduced sensitivity was demonstrated with the RIDA® QUICK N1403 for GI (Ambert-Balay and Pothier, 2013; Battaglioli et al., 2012) and with the RIDA® QUICK N1402 for genotype GII.17, a new emerging genotype (Théry et al., 2016). This drawback has to be balanced with the benefits of ease of use, availability and speed, applied to the specific situation of the laboratory. When there is a daily availability of molecular tests, there seems to be limited value of a rapid antigen test. However, in laboratories were molecular tests are not readily available, an antigen test can be of great value, despite its lower sensitivity.

Although the RIDA® QUICK antigen test, and norovirus antigen tests in general, exhibit a lower sensitivity compared to molecular tests, 15 (12%) of 125 positive stool samples in our study were detected by RIDA® QUICK antigen testing but not by RIDA® GENE norovirus I and II real-time RT-rtPCR (PG1415). Those samples were also mostly not detected by the RIDA® GENE Norovirus detection kit (PG1405) and the Xpert® Norovirus assay. By doing a more in depth molecular analysis using primers targeting another region in the genome, the samples could be confirmed as norovirus. By using the in-house SYBR®Green

Table 3
Performance characteristics of the RIDA® QUICK on liquid and loose stool samples ($n = 771$) for the different study sites (S). A sample was considered norovirus positive if at least one of the RT-rtPCR's was positive (RIDA® GENE Norovirus I and II (PG1415), RIDA® GENE Norovirus detection kit (PG1405), Xpert® Norovirus assay or in-house SYBR®Green PCR).

Study site and result for assay	RT-rtPCR result		% Positivity	% Sensitivity	% Specificity	% PPV	% NPV	κ value[a]
	(number of samples)							
	Positive	Negative	(95% CI)	(95% CI)	(95% CI)	(95% CI)	(95% CI)	(95% CI)
S1 ($n = 96$)								
Positive	7	0	7.3	100	100	100	100	1.00
Negative	0	89	(3.0, 14.5)	(59.0, 100)	(95.9, 100)	(59.0, 100)	(95.9, 100)	(1.00, 1.00)
S2 ($n = 141$)								
Positive	16	0	18.4	61.5	100	100	92.0	0.72
Negative	10	115	(12.4, 25.8)	(40.6, 79.8)	(96.8, 100)	(79.4, 100)	(85.8, 96.1)	(0.56, 0.88)
S3 ($n = 80$)								
Positive	11	2	15.0	91.7	97.1	84.6	98.5	0.86
Negative	1	66	(8.0, 24.7)	(61.5, 99.8)	(89.8, 99.6)	(54.6, 98.1)	(92.0, 100)	(0.70, 1.00)
S4 ($n = 246$)								
Positive	48	1	26.0	75.0	99.5	98.0	91.9	0.81
Negative	16	181	(20.7, 32.0)	(62.6, 85.0)	(97.0, 100)	(89.2, 100)	(87.2, 95.3)	(0.72, 0.89)
S5 ($n = 208$)								
Positive	9	0	7.7	56.3	100	100	96.5	0.70
Negative	7	192	(4.5, 12.2)	(29.9, 80.3)	(98.1, 100)	(66.4, 100)	(92.9, 98.6)	(0.50, 0.91)
Total ($n = 771$)								
Positive	91	3	16.2	72.8	99.5	96.8	95.0	0.80
Negative	34	643	(13.7, 19.0)	(64.1, 80.4)	(98.7, 99.9)	(91.0, 99.3)	(93.1, 96.5)	(0.74, 0.86)

S1: HH Hospital (Lier); S2: Imelda Hospital (Bonheiden); S3: Oost-Limburg Hospital (Genk); S4: Jan. Yperman Hospital (Ypres) S5: OLV Hospital (Aalst).
[a] weighted kappa (linear weights).

PCR which only used the primers QNIF2$_{5012}$/COG2R$_{5100}$, described in the ISO/TS15216:2012, and not the probe QNFIS$_{5042-5061}$, it was possible to detect norovirus in these samples. Our hypothesis of lacking a fragment in the ORF1-ORF2 junction was confirmed by the fact that by using the G2SKF$_{5046}$/G2SKR$_{5389}$ primers, for amplifying the capsid region, also no amplification product could be obtained. A similar phenomenon has recently been described in the RT-rtPCR-based detection of norovirus GII.4 Sydney (Zhuo et al., 2015). A single nucleotide polymorphism at the probe-binding site impeded the detection, resulting in a lowered sensitivity of the RT-rtPCR. Continuous genetic evolution gives rise to new variants of norovirus and poses a real challenge for accurate identification of all these variants. This has also been described for Influenza virus. Due to evolutionary drift in influenza, a reduced sensitivity of some commercial PCR detection kits was seen (Huzly et al., 2016). These findings highlighted the need of a continuous monitoring of test performance of available PCR assays. A multi-gene targeting PCR based system could overcome this problem for detecting new variants circulating in the population (Steensels et al., 2013). As a consequence of the suboptimal performance of the RIDA® GENE as gold standard in our study, the sensitivity of the RIDA® QUICK assay might be slightly overestimated.

Although no outbreak was noticed in the participating study sites, which is also supported by the heterogeneity of genotypes found in every center, a marked difference in prevalence of norovirus was noticed among the different centers. This could be explained by a difference in age distribution in the different centers with higher prevalence when more children were included. A recent study considering the results obtained from norovirus incidence data in the European Union revealed that virtually all children will have suffered at least one episode of norovirus illness by age 5, one in 7 children will have required a medical visit, one in 98 will have been hospitalized and one in 51.000 will have died (Kowalzik et al., 2015). There was also a striking difference in sensitivity of the RIDA® QUICK between the participating centers. A possible explanation would be the limited number of positive samples in some centers. Also, a difference in viral load of positive samples was found between the different centers, with the highest viral loads for the center with the highest sensitivity. However, a more fundamental reason could also be at the basis. A potential disadvantage of visually read rapid antigen tests is that some level of interpretation is required from the test operator and that classification of positive versus negative results can be quite subjective, especially for samples with a lower viral load where the test line can be very faint. An automatic reader or
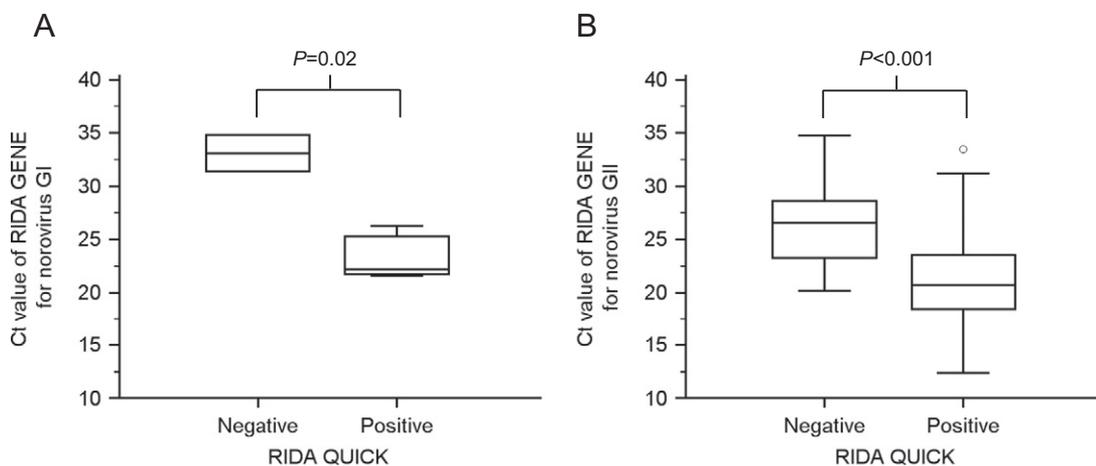

Fig. 1. Comparison of Ct values of the RIDA® GENE PCR between RIDA® QUICK positive and negative samples for norovirus GI (A, $n = 5$) and norovirus GII (B, $n = 105$). False negative samples had a significantly higher Ct value (lower viral load) compared to true positive samples.

independent reading by different well-trained technicians could potentially overcome this issue.

The most frequently detected norovirus genotype in this study was GII.4 which corresponds with the results of previous studies performed in Belgium (Mathijs et al., 2011; Wollants et al., 2015). In the study period 2014–2015, the GII.4 Sydney 2012 pandemic variant circulated in patients from all the hospital sites participating in this study. The new described GII.17 genotype emerging since the winter of 2014/2015 was not found in the population at that moment (de Graaf et al., 2015). The RIDA® QUICK positive/RIDA® GENE negative samples concerned norovirus variants of the genotype GII. P4 and in one sample genotype GII. Pe. It is not known which genotype is present at the capsid region to conclude if these stains are a result of a mutation in the genome or if they are new recombinants. Whole genome sequencing could give a final answer to this (Bavelaar et al., 2015).

In conclusion, the updated RIDA® QUICK N1402 can be used as a reliable test for rapid confirmation of norovirus in stool samples. However, a negative result does not exclude norovirus infection.

Acknowledgements

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