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Evaluation of RIDA®GENE norovirus GI/GII real time RT-PCR using stool specimens collected from children and adults with acute gastroenteritis☆

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ABSTRACT

Background: Norovirus is the leading cause of epidemic and sporadic acute gastroenteritis (AGE) in the United States. Widespread prevalence necessitates implementation of accurate norovirus detection assays in clinical diagnostic laboratories.
Objective: To evaluate RIDA®GENE norovirus GI/GII real-time RT-PCR assay (RGN RT-PCR) using stool samples from patients with sporadic AGE.
Study design: Patients between 14 days to 101 years of age with symptoms of AGE were enrolled prospectively at four sites across the United States during 2014–2015. Stool specimens were screened for the presence of norovirus RNA by the RGN RT-PCR assay. Results were compared with a reference method that included conventional RT-PCR and sequencing of a partial region of the 5′end of the norovirus ORF2 gene.
Results: A total of 259 (36.0%) of 719 specimens tested positive for norovirus by the reference method. The RGN RT-PCR assay detected norovirus in 244 (94%) of these 259 norovirus positive specimens. The sensitivity and specificity (95% confidence interval) of the RGN RT-PCR assay for detecting norovirus genogroup (G) I was 82.8% (63.5–93.5) and 99.1% (98.0–99.6) and for GII was 94.8% (90.8–97.2) and 98.6% (96.9–99.4), respectively. Seven specimens tested positive by the RGN-RT PCR that were negative by the reference method. The fifteen false negative samples were typed as GII.4 Sydney, GII.13, GI.3, GI.5, GI.2, GII.1, and GII.3 in the reference method.
Conclusions: The RGN RT-PCR assay had a high sensitivity and specificity for the detection of norovirus in stool specimens from patients with sporadic AGE.

1. Background

Globally, norovirus contributes to 18% of all diarrheal diseases and is associated with approximately 200,000 deaths each year [1]. Individuals from all age groups can be infected by norovirus [2]. Recent studies indicate that with the introduction of rotavirus vaccines (70–80% uptake), norovirus has become the leading etiology for acute gastroenteritis (AGE) among children in the United States [3–5]. Although norovirus gastroenteritis is for most people a self-limiting disease, it may be life-threatening for young children, the elderly, and immunocompromised patients [2].

Noroviruses are a genetically and antigenically diverse group of viruses belonging to the family *Caliciviridae* [6]. They can be classified into at least six established and one recently proposed genogroups (GI-GVII) with more than 40 genotypes [1,2,6,7]. GI and GII viruses are the leading cause of infections in humans over the last decade. Specifically, GII.4 viruses are responsible for the majority of norovirus infections both globally and in the USA [8,9]. Enzyme immunoassays and rapid

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Table 1
Patient statistics of participating sites.

Location[a]	Children[b]	Adults[b]	Male/Female	GI[c]	GII[c]
KC Metropolitan area; MO & KS	249	0	144/105	16	55
Albany, NY	11	88	51/48	11	60
Los Angeles, CA	0	215	208/7	2	3
Lansing, MI	6	150	44/112	0	112
Total	266	453	447/272	29	230

[a] MO: Missouri; KS: Kansas; CA: California; MI: Michigan.
[b] The age cut-off for children and adults was 18 years.
[c] GI and GII positive detections using composite reference method.

immuno-chromatographic assays have been developed but sensitivity could range between 31.6%–92.0% and 17.0%–90.2%, respectively [2]. PCR based methods are currently considered the gold standard method for norovirus detection [2].

The RIDA®GENE norovirus GI/GII real-time RT-PCR assay (RGN RT-PCR; R-Biopharm AG, Darmstadt, Germany) can simultaneously detect and differentiate norovirus GI and GII in stool specimens. This RGN RT-PCR targets the relatively conserved ORF1/ORF2 junction region. Although few groups internationally have evaluated the RGN RT-PCR [10,11], an assessment of the assay in the United States has not been reported.

The primary goal of this study was to evaluate the performance characteristics of RIDA®GENE norovirus GI/GII RT-PCR assay using stool samples from pediatric and adult patients with AGE.

2. Materials and Methods

Study design and sample collection: This study was conducted at four sites including two primary care medical facilities and two public health laboratory and a reference site (Centers for Disease Control and Prevention [CDC]) from February 2014 to December 2015 (Table 1). The study protocol was approved by the respective institutional review boards. Specimens came from male and female patients between the ages of 14 days to 101 years. Individual's greater than 18 years were considered adults for study analysis purposes. Two enrollment site prospectively enrolled subjects with symptoms of AGE during their visit to the health care facilities. Written consent was obtained and stool specimens were collected exclusively for this study. The other two facilities (public health laboratories) exclusively enrolled left-over stool specimens that were submitted for gastroenteritis testing for routine clinical care (Table 1). Stool specimens were obtained within ten days from onset of AGE symptoms for all 4 sites. Specimens were stored at 2–8 °C until nucleic acid was extracted for performing RGN RT-PCR within a week of sample collection. Stool specimens were also tested at the reference site by a reference method/algorithm, comprised of conventional RT-PCR of region C and retesting of the region C negative samples by conventional RT-PCR of region D, followed by sequencing of the PCR products of appropriate sizes [12–14]. Sequences were compared to CDC reference databases for genotype determination.

Sample preparation: Unpreserved raw stool specimens were well mixed and homogenized (if necessary by adding few drops of deionized water). Samples were divided into 250 µl aliquots. The first aliquot was shipped on cold packs to the CDC for testing with the reference method. The second aliquot was extracted and tested in the RGN RT-PCR assay as per manufacturer's protocol. The remainder of the raw stool specimens were kept at 4 °C until testing was completed.

RNA extraction and RIDA®GENE norovirus GI/GII PCR assay: The raw stool specimen was diluted 1:10 with water, the suspension was homogenized, and the homogenate was centrifuged for 1 min at 13,000 × *g*. Two hundred microliters of clear supernatant and 20 µl of internal control RNA (bacteriophage MS2, provided with the kit) was utilized for nucleic acid (NA) extraction using the NucliSENS® easyMAG® instrument (bioMérieux Inc., Durham, NC, USA) to obtain 60 µl elution volume. A negative extraction control was included in each run. NA was stored at 4 °C for up to 8 h. If RT-PCR was not performed within 8 h, NA was stored at −20 °C for up to a week. After RT-PCR testing was completed, NA was stored at −80 °C.

Applied Biosystems®7500 FAST Dx System (Thermo Fisher Scientific Corp., MA, USA) with software version 1.4 was utilized for the one-step RGN RT-PCR assay. The reaction mixture consisted of 19.4 µl of reaction mix, 0.7 µl of enzyme mix, and 5 µl of NA or controls provided in the kit. The thermal profile included cDNA synthesis at 58 °C for 10 min; denaturation at 95 °C for 5 min; amplification with 45 cycles of 95 °C for 15 s, and 55 °C for 30 s; and cooling at 40 °C for 10 s. The assay utilized Taqman® probes with fluorescent reporter dye (norovirus GI: Cy5, norovirus GII: FAM, and internal control RNA: VIC) for detection of the amplified targets.

Statistical analysis: Overall prevalence of norovirus as well as age, gender and symptom distribution among the norovirus-positive and -negative groups were determined.

Two by two data tables were used to determine the sensitivity, specificity, positive and negative predictive values, of the RGN RT-PCR assay in comparison with the reference method. The true positive, true negative, false positive, and false negative categories were determined based on the reference method/algorithms. Estimates are presented together with their 95% confidence intervals (CI) as calculated from site: http://vassarstats.net/clin1.html. The median, minimum and maximum C_T values for both GI and GII were determined.

3. Results

A total of 769 samples were collected by the four study sites. Of these, 50 had to be excluded due to missing results or other exclusion criteria (extended time span or insufficient sample volume n = 24, missing age or gender information n = 6, exceeded expiry of extraction kit n = 19, no result for reference method n = 1). Tests for nine patient samples were repeated because the original test result was invalid due to internal control failure (Invalid Rate: 1.3%). The 719 study samples included 447 specimens from males and 272 from females. The median patient age was 52 years (range 14 days-101 years). The number of patients with diarrhea only, vomiting only, and both diarrhea and vomiting were 294 (40.9%), 109 (15.2%), and 273 (37.9%), respectively. Detailed patient demographic information is listed in Table 2.

Reference method and the experimental method detected norovirus in 259 (36%) and 251 (34.9%) of the 719 tested specimens. The sensitivity of the RGN RT-PCR assay compared to the composite reference method for detection of GI and GII was 82.8% (95% CI: 63.5–93.5) and 94.8% (95% CI: 90.8–97.2), respectively. The specificity of the RGN RT-PCR assay for GI and GII was 99.1% (95% CI: 98.0–99.6) and 98.6% (95% CI: 96.9–99.4), respectively. The overall sensitivity and specificity of RGN RT-PCR for norovirus detection was 94.2% (95% CI: 90.4–96.6) and 98.5% (95% CI: 96.7–99.3), respectively. The RGN RT-PCR assay detected dual infections with both GI and GII in four specimens; the reference method confirmed 3 specimens as positive for GII and one as positive for GI. There were two additional specimens (one G1 and one GII) for which the genogroups detected by the RGN RT-PCR assay did not match those detected by the reference method. Detailed RGN RT-PCR assay performance parameters for all study subjects and specific parameters for adult and pediatric population are listed in Table 3.

Fifteen specimens negative by the RGN RT-PCR assay were positive by the reference method. These included seven different genotypes: GII.4 Sydney (n = 6), GII.13 (n = 3), GI.3 (n = 2), GI.5 (n = 1), GI.2 (n = 1), GII.1 (n = 1), and GII.3 (n = 1). Seven (2 GI, 5 GII) specimens that were negative by the reference method tested positive by RGN RT-PCR; the C_T value for the two GI false positive results were 27.5 and 29.9 and the median C_T value of GII false positive results was 29.7 (range 17.0–35.9).

Overall, of the 259 specimens determined positive by the reference method, 29 (11.2%) were found to be GI and 230 (88.8%) were found