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Research Article

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Evaluation of an Assay Based On Multiple Detection Temperature Technique for Simultaneous Detection of Viral Gastroenteritis-Causing Pathogens

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Abstract

Background: Multiple detection temperature (MuDT) technique is an advanced method for the analysis of multiple Ct (cycle threshold) values in a single channel.
Objectives: The advantage of this method has been shown only in DNA samples, restricting its diagnostic applicability. This technique was evaluated in this study for its efficacy in the analysis of target RNA.
Methods: Allplex GI-virus assay was developed to detect pathogens causing viral gastroenteritis, one of the major diseases caused by RNA viruses. This one-step multiplex real-time polymerase chain reaction (PCR) based on the MuDT technique permits simultaneous amplification and detection of target nucleic acids of norovirus GI, norovirus GII, rotavirus A, adenovirus F, astrovirus, and sapovirus genogroups. The assay was tested for analytical sensitivity, cross-reactivity, repeatability, and applicability to clinical samples.
Results: The analytical performance was validated for each target. The assay demonstrated high analytical sensitivity and no cross-reactivity, and the repeatability tests showed excellent performance with high accuracy. Analytical performance validation indicated high positive agreement and negative agreement for this method. In the analyses comparing Allplex GI-virus Assay and commercial Seeplex Diarrhea-V ACE Detection using clinical specimens, the positive and negative agreements between the test results were found to be 94.9% and 98.8%, respectively. Statistical analysis showed that there was no difference in the performance between the two products.
Conclusions: The Allplex GI-virus Assay can rapidly detect six viruses in a single tube without the complementary DNA synthesis step, and this assay was shown to represent an improved molecular diagnostic tool for the simultaneous detection of several RNA viruses. Therefore, our results suggest that the MuDT technique may represent a new molecular diagnostic method for the detection of RNA viruses.

Keywords: Diagnosis, Real-Time Polymerase Chain Reaction, RNA, Viral, Molecular Diagnostic Techniques

1. Background

Medical diagnostic methods currently include genetic tests that use blood samples, microbial cultures, blood transfusion tests, as well as cytopathologic diagnostic procedures. In addition to advancements in modern medicine, diagnostic improvements have also been achieved (1, 2). Molecular diagnostic methods using multiplex and real-time polymerase chain reaction (PCR) have advanced rapidly for the detection of causative pathogens, and they have been used for the diagnosis of various infectious diseases. Following their validation, it has been demonstrated an equality between the novel molecular diagnostic tests and the already existing methods (3-6). Although various real-time PCR techniques have been developed and introduced to date, the number of the targets that they are able to detect is limited due to the difficulty of detection of multiplex targets in a single channel (7).

In 2012, the Tagging Oligonucleotide Cleavage and Extension (TOCE) technique, which is able to detect multiple targets in a single channel based on melting temperature analysis, was developed (8). The multiple detection temperature (MuDT) technique, introduced in 2014, is used to analyze the difference between fluorescence signals from TOCE-generated oligonucleotides amplified during the annealing and extension steps. This enables the detection of multiple targets in one channel using their individual Ct (cycle threshold) values, therefore overcoming the target number limitation (9). The MuDT technique has only been shown to be effective for DNA targets, and its applicability for the detection of RNA viruses has not been examined.

RNA viruses that can be detected using molecular diagnostic tools include viruses causing meningitis, respiratory infections, and gastroenteritis (10). Gastroenteritis is

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known to occur mostly in infants and toddlers, but it can also occur in adults. Its outbreaks occur regardless of season, and gastroenteritis has become a major health issue worldwide (11, 12). Viral gastroenteritis symptoms include headache, fever, and vomiting, all within 12 - 48 hours after exposure, and these symptoms improve within 14 days at most. In some cases of severe diarrhea, hematochezia, diarrhea outbreak, or diarrhea in patients with weak immunity, rapid detection of the infection source is crucial for the determination of therapeutic approaches, such as the administration of antibacterial agents, and the prevention of spreading of infectious diarrhea (12).

The estimated number of acute gastroenteritis patients worldwide is 300 - 500 million annually, and the yearly death toll was estimated to be approximately 2 million patients (13). Gastroenteritis spreading occurs mostly by the intake of contaminated water or food, and the known major causative viruses include norovirus genogroups, group A rotaviruses, astroviruses, enteric adenoviruses, and sapoviruses (14). The main aim of molecular diagnostic method research for the detection of viral gastroenteritis is the development of one-step real-time PCR from multiplex PCR (15-20). Previously developed PCR methods all require gel electrophoresis steps or PCR reactions that are performed after reverse transcription.

2. Objectives

In this study, Allplex GI-Virus Assay was developed for the simultaneous detection of six major enteric viruses with the one-step real-time PCR procedure in a single reaction using the MuDT technique, and its performance and ability to target RNA viruses were validated. Additionally, the performance of this assay was compared with that of a commercially available multiplex PCR assay.

3. Methods

3.1. Clinical Specimens

This prospective study was approved by the institutional review board of Seegene medical foundation, South Korea (IRB No SMF-IRB 2015004), and it was performed in accordance with the Helsinki Declaration of 1975, as revised in 1983. In total, 1489 stool specimens were collected from July 2015 to November 2015. Each specimen was immediately stored at -70°C until further simultaneous analyses.

3.2. Multiplex PCR

To evaluate the quality of the real-time one-step PCR assay, Seeplex Diarrhea-V ACE Detection (DR6411Y, Seegene, Seoul, South Korea), a commercial multiplex viral gastroenteritis assay, was used for comparison. This assay is designed for the detection of the Norovirus GI (NVGI), Norovirus GII (NVGII), Rotavirus A (ROV), Adenovirus F (ADV-F; Serotype 40/41), and Astrovirus (ASV). It consists of reverse transcription and PCR amplification steps, followed by the resolution and detection of amplified DNA products by capillary electrophoresis. Complementary DNA (cDNA) synthesis was performed with 8 μL of extracted nucleic acids using a first strand cDNA synthesis kit (Fermentas, Ontario, Canada), according to the manufacturer's instructions. The thermal cycling conditions consisted of an initial activation of 15 minutes at 94°C, followed by 40 cycles of 30 s at 94°C, 90 s at 60°C, and 90 s at 72°C. This process was performed using an Applied Biosystems GeneAmp 9700 system (Life Technologies, Carlsbad, CA, USA). The amplification products were detected using agarose gel electrophoresis.

3.3. Nucleic Acid Extraction

Nucleic acid molecules were extracted from 200 μL of each sample using QIAamp DSP DNA Mini Kit (Qiagen, Hilden, Germany), which can be used to extract viral RNA as well. For the Allplex GI-virus Assay, bacteriophage MS2 was added to each sample as an internal control, according to the manufacturer's instructions.

3.4. In Vitro Transcription

Rotavirus A, Astrovirus and sapovirus (SV) were amplified using the primers for the Allplex GI-virus Assay (GI9701Y, Seegene, Seoul, South Korea). The thermal cycling conditions consisted of an initial activation for 15 min at 94°C, followed by 40 cycles of 30 s at 90°C, 90 s at 60°C, and 90 s at 72°C. The reaction was performed using an Applied Biosystems GeneAmp 9700 system (Life Technologies, Carlsbad, CA, USA). Each PCR product was cloned into a TOPO TA vector (Invitrogen, Carlsbad, CA, USA) containing the T7 polymerase promoter. The complete inserts, including the T7 promoter, were amplified with vector-specific primers. The PCR products were purified and transcribed in vitro using the MegaScript T7 in vitro transcription kit (Ambion, TX, USA), and the obtained RNAs were purified using MEGAclear kit (Ambion, TX, USA). The isolated RNA samples were quantified spectrophotometrically and the copy numbers were calculated.

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3.5. One-step Multiplex Real-time PCR Based on the MuDT Technique

The Allplex GI-virus Assay (Seegene, Seoul, South Korea) is a one-step multiplex real-time PCR that permits simultaneous amplification and detection of target nucleic acids of NVGI, NVGII, ROV, ADV-F, ASV, and SV (genogroups G1, 2, 4). For this assay, one-step real-time PCR was performed with 5 μL of nucleic acid extract, using a CFX96 PCR detection system (Bio-Rad, Hercules, CA, USA) under the following conditions: 20 minutes at 50°C and 15 minutes at 95°C, followed by 45 cycles of 95°C for 10 seconds, 60°C for 1 minute, and 72°C for 30 seconds. To analyze two targets in one channel, the fluorescence intensity was measured at 60°C and 72°C. The analysis was performed using the designated software (Seegene Viewer, Seegene, Seoul, South Korea).

3.6. SV Sequencing

Sequencing was performed using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Forster City, CA, USA) on an ABI PRISM 3730XL analyzer (Applied Biosystems, Forster City, CA, USA). The obtained sequences were compared with the reference sequences through NCBI BLAST, using the GenBank database (NIH, Bethesda, MA, USA).

3.7. Determination of the Detection Limit of the One-step Multiplex Real-Time PCR Assay

The limit of detection (LoD) range was first estimated and then, a preliminary LoD study was performed for each culture fluid (ZeptoMetrix Corporation, Buffalo, NY, USA) or in vitro transcribed RNA. NVGI (0810086CF), NVGII (0810087CF), and ADV-F (0810085CF) were purchased from ZeptoMetrix. NVGI and NVGII represent recombinant culture fluids.

Culture fluid viral stocks were used in a series of six 10-fold dilutions in a negative matrix, and in vitro transcribed RNAs were used in a series of five 10-fold dilutions in TE buffer. The test was performed using three Allplex GI-virus lots with four replicate tests for each lot. Afterward, the analytical sensitivity of the assay was determined. The results obtained in the LoD estimation study were used to determine the range for the LoD analysis. The last dilution with a 100% detection rate was used to prepare either five or four dilutions that were tested on three Allplex GI-virus Assay lots, in replicates of eight. The results obtained using the three lots were combined, and the detection rate was calculated for each dilution. LoD was defined as the lowest concentration of the target that was detected $\geq$ 95% of the time.

3.8. Cross-Reactivity with Common Enteric Bacteria, Viruses, and Protozoa

Cross-reactivity with bacteria, viruses, and protozoa was examined using nucleic acids isolated from bacteria and protozoa. The control isolates were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), ZeptoMerix (ZMC, Buffalo, NY, USA), Korean Collection for Type Culture (KCTC, Daejeon, South Korea), Korean Culture Center of Microorganisms (KCCM, Seoul, South Korea), the Biological and Emerging Infections Resources Program (BEI Resources, Manassas, VA, USA), and National Culture Collection for Pathogens (NCCP, New York, NY, USA). The cross-reactivity of the Allplex GI-virus Assay was assessed using 38 species of different bacteria, viruses, and protozoa.

3.9. Repeatability Study

Nineteen different samples were prepared consisting of one negative sample and 18 stimulated analytes, which included moderately positive (3 $\times$ LoD, positive results obtained 100% of the time), minimally positive (1 $\times$ LoD, positive results obtained approximately 95% of the time), and highly negative samples (0.5 $\times$ LoD, negative results obtained approximately 20% to 80% of the time). The repeatability of the test was assessed using the data obtained from the LoD calculations. Repeatability was tested using the known positive samples in three different concentrations and a negative control for each target. Each target was tested twice a day for a total of 20 days by a single operator, and duplicates of each panel sample were tested per each run. Within-run, run-to-run, and day-to-day CV% (coefficient of variation) values were analyzed, and the accuracy of the test for each product was evaluated.

3.10. Statistical Analysis

Statistical analyses were performed using SPSS software, version 18 (SPSS Inc., Chicago, IL, USA) and an online tool for assessing statistical agreement (www.john-uebersax.com/stat/raw.htm). Cohen's kappa value was calculated and used to compare the capacity of viral strain detection between the Allplex GI-virus Assay and Seeplex Diarrhea-V ACE Detection.

4. Results

4.1. Analytical Sensitivity

To examine the analytical performance of MuDT for RNA detection, the Allplex GI-virus Assay was developed and its sensitivity was tested using previously determined amounts of titrated NVGI, NVGII, and ADV-F strains. For ROV, ASV, and SV, the titrated strains were commercially

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unavailable, and their DNAs were first converted to RNAs through in vitro transcription and then quantified. The amount of each 10-fold diluted target was estimated, concentration intervals were subdivided focusing on the intervals in which the detection was interrupted, tests were repeated 24 times, and the concentrations of the samples for which the viruses were detected at least 23 times were determined (positive result rate > 95%). The amounts of NVGI, NVGII, and ADV-F were determined to be 75, 5, and 0.5 $TCID_{50}$/mL, respectively, whereas the quantities of ROV, ASV, and SV were found to be 50, 500, and 5000 RNA copies/reaction, respectively (Table 1).

4.2. Cross-Reactivity

Specificity was tested using a total of 38 strains, including eight protozoan, six viral, and 24 bacterial species (Table 2). No amplification of real-time PCR products was detected for any of the investigated strains, indicating that this assay has no cross-reactivity with other strains.

4.3. Repeatability

The obtained coefficients of variation were 1.98% - 4.66% for within-run, 1.89% - 4.33% for run-to-run, and 0.93% - 2.89% for day-to-day repeatability, showing less than 5% variation in all analytical conditions for all targets, which demonstrates the excellent repeatability of this assay (Table 3).

4.4. Comparison of the Allplex GI-Virus Assay with the Seeplex Diarrhea-V ACE Detection

To validate the performance of the MuDT technique, the Seeplex Diarrhea-V ACE Detection, a commercial diagnostic test, was used for comparative tests using clinical specimens. The obtained results are presented in Table 4. In total, 1489 specimens were tested. SV, which is undetectable by the Seeplex Diarrhea-V ACE Detection, was excluded from the statistical analysis. Each obtained result was analyzed according to the specific analyte to enable each co-infection specimen to be considered an independent positive target. In total, 7445 results were statistically analyzed. The number of the analyzed positive samples was 296 for the Allplex GI-virus Assay and 290 for Seeplex Diarrhea-V ACE Detection, and the overall positive agreement was shown to be 94.9% (95% CI, 93.1 - 96.7), whereas the positive agreement for each target was $\geq$ 88.4% (NVGI, NVGII, ADV-F, ROV, and ASV). The overall negative agreement was determined to be 98.8% (95% CI, 98.3 - 99.2), and the negative agreement for each target (NVGI, NVGII, ADV-F, ROV, and ASV) was $\geq$ 99.6%. Cohen's kappa value was determined to be 0.95 (95% CI, 0.92 - 0.96), indicating an almost perfect agreement between the results, and P = 0.362 was obtained by McNemar's test, indicating that there was no significant difference in performance between the two assays. The specimens showing discrepant results in these two assays were further analyzed using type-specific PCR and direct sequencing (Table 5).

4.5. Correlation of One-Step Multiplex Real-Time PCR Results with SV Sequencing

Using the Allplex GI-virus Assay, we were able to detect 19 SV-positive samples out of 1489 analyzed stool specimens, and the positive samples were sequenced to confirm these results. The samples were confirmed to be SV, which demonstrated that this test is also able to detect SV.

5. Discussion

To demonstrate the ability of the MuDT method to detect RNA viruses, the Allplex GI-virus Assay, which is able to target viruses causing viral gastroenteritis, was developed and its analytical performance was investigated by testing its sensitivity, specificity, and repeatability. We have not provided the information regarding the oligonucleotides used in this assay, as this is a commercial product in development. All investigated properties of this assay were shown to be comparable to those of a commercial molecular diagnostic kit. The obtained sensitivity results for NVGI, NVGII, and ADV-F showed a 100% detection rate for at least five-fold of the LoD, and the correlation coefficient (R^2) was determined to be $\geq$ 0.99 for all targets (data not shown). These values were similar to the values (NG gDNA $R^2 > 0.99$) obtained in previous studies that investigated the MuDT technique using DNA targets (9), demonstrating that the one-step MuDT technique targeting RNA has a rate of performance similar to that of the viral DNA targeting.

Additional investigations were conducted to demonstrate the clinical performance of this assay by comparing with a commercial kit (21). Seeplex Diarrhea-V ACE Detection, using clinically obtained stool specimens, was used to detect NVGI, NVGII, ROV, ADV-F, and ASV. We determined that the results obtained by the Allplex GI-virus Assay were comparable to the results obtained using this commercial kit. These results showed the highest detection rate for NV, followed by ROV. In contrast, the detection rates of ASV and ADV-F were below 2%. This is consistent with the results of a previous study showing that NV and ROV are among the most common causative viruses in acute diarrhea (22). Statistical analysis of the total positive and negative rates of detection in stool specimens was performed, and the results obtained for each analyte were analyzed separately, resulting in a total of 7745 parent populations. In total, 278 specimens were identified as positive using both assays, whereas 7137 specimens were shown to be negative.

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Table 1. Analytical Sensitivity of the Allplex GI-virus Assay

Concentration (TCID50/mL)	POS/Total	POS Rate (%)	95% CI	Concentration (Copies/Reaction)	POS/Total	POS Rate (%)	95% CI
NVGI				**ROV**			
100	24/24	100	86.20 - 100.00	100	24/24	100	86.20 - 100.00
75	24/24	100	86.20 - 100.00	75	24/24	100	86.20 - 100.00
50	19/24	79.2	59.50 - 90.80	50	24/24	100	86.20 - 100.00
25	17/24	70.8	50.80 - 85.10	25	20/24	83.3	64.15 - 93.32
10	4/24	16.7	6.70 - 35.90	10	18/24	75	55.10 - 88.00
NVGII				**ASV**			
25	24/24	100	86.20 - 100.00	1000	24/24	100	86.20 - 100.00
10	24/24	100	86.20 - 100.00	750	24/24	100	86.20 - 100.00
7.5	24/24	100	86.20 - 100.00	500	23/24	95.8	79.76 - 99.26
5	23/24	95.8	79.76 - 99.26	250	19/24	79.2	59.53 - 90.76
2.5	16/24	39.3	6.68 - 35.85	100	17/24	70.8	50.83 - 85.09
ADV-F				**SV**			
10	24/24	100	86.20 - 100.00	10000	24/24	100	86.20 - 100.00
5	24/24	100	86.20 - 100.00	7500	24/24	100	86.20 - 100.00
1	24/24	100	86.20 - 100.00	5000	24/24	100	86.20 - 100.00
0.5	24/24	100	86.20 - 100.00	2500	22/24	91.7	74.15 - 97.68
0.1	16/24	66.7	46.71 - 82.03	1000	4/24	8.3	6.68 - 35.85

Abbreviations: NVGI, norovirus GI; NVGII, norovirus GII, ADV-F, adenovirus F; ROV, rotavirus; ASV, astrovirus; SV, sapovirus; CI, confidence interval.

Seeplex Diarrhea-V ACE Detection had a higher detection frequency for NVGII and ASV, whereas the other investigated viruses were more often detected using the Allplex GI-virus Assay.

Seeplex Diarrhea-V ACE Detection could identify 12 positive samples that had not been detected by the Allplex GI-virus Assay, whereas the Allplex GI-virus Assay detected 18 positive samples that were not detectable by Seeplex Diarrhea-V ACE Detection. Cohen's kappa value was determined to be 0.95, indicating an almost perfect agreement between the obtained results. McNemar's test showed a P value of 0.362, further confirming that there is no significant difference in performance between these products. Additionally, whenever the results obtained by the two investigated assays were inconsistent, the samples were sequenced. Eighteen samples positive for each target in the Allplex GI-virus Assay were sequenced, whereas nine samples determined to be positive by the Seeplex Diarrhea-V ACE Detection could not be sequenced because of the low yield of the PCR product.

All results were consistent with the test results obtained using the Allplex GI-virus Assay, and the inconsistency may have resulted from the types of viruses detectable by this assay at the time when the product was developed. Furthermore, the internal control of the Seeplex Diarrhea-V ACE Detection can be detected only in the PCR step, and reverse transcription, PCR analysis, and electrophoresis are necessary to obtain the results, which is time-consuming and complicated. In contrast, the Allplex GI-virus Assay has been designed to detect the internal control sample through all processes from the RNA extraction to PCR, enabling quality control at the extraction step. This assay was demonstrated to carry a lower risk of contamination and it requires a shorter procedure time because it incorporates one-step reverse transcription and PCR process.

Viral gastroenteritis is highly contagious and can cause problems in different populations; therefore, it is critical to identify the causative pathogens. Despite the existence of various detection methods, including PCR analysis following reverse transcription and one-step real-time PCR, the number of targets that can be detected at the same time is limited. The detection limit for multiple targets can be overcome by the fluorescent bead method. However, the determination of positivity or negativity of the samples by hybridization following the PCR reaction is a disadvantage of these methods (23). In contrast, it was demon-

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Table 2. Cross-Reactivity Results

Organism	Source	Result	Organism	Source	Result
Aeromona scaviae	ATCC	ND	*Herpes simplex* virus type 1	ZMC	ND
Aeromonas salmonicida* subsp. *masoucida	KCCM	ND	*Herpes simplex* virus type 2	ZMC	ND
Aeromonas veronii* bv *veronii	ATCC	ND	*Salmonella bongori*	KCCM	ND
Blastocystis hominis	ATCC	ND	*Salmonella choleraesuis* subsp. *Arizonae*	KCCM	ND
Campylobacter coli	KCTC	ND	*Salmonella choleraesuis* subsp. *choleraesuis*	KCCM	ND
Campylobacter jejuni* subsp. *jejuni	KCTC	ND	*Salmonella choleraesuis* subsp. *diarizonae*	KCCM	ND
***Clostridium difficile* NAP1**	ATCC	ND	*Salmonella enterica* subsp. *enterica serovar Paratyphi C*	KCCM	ND
Clostridium difficile	ATCC	ND	*Salmonella enteritidis*	KCCM	ND
Cryptosporidium hominis	BEI Resources	ND	*Salmonella houtenae*	Clinical isolate	ND
Cryptosporidium meleagridis	BEI Resources	ND	*Salmonella typhimurium*	KCCM	ND
Cryptosporidium parvum	ATCC	ND	*Shigella boydii*	KCCM	ND
Cyclospora cayetanensis	Clinical isolate	ND	*Shigella dysenteriae*	NCCP	ND
Cytomegalovirus	ATCC	ND	*Shigella flexneri*	KCCM	ND
Dientamoeba fragilis	Clinical isolate	ND	*Shigella sonnei*	KCCM	ND
Entamoeba histolytica	ATCC	ND	*Varicella Zoster* virus	ZMC	ND
Enterovirus type 71	ZMC	ND	*Vibrio cholerae* Z132	ZMC	ND
***Escherichia coli* (Enterotoxigenic *E. coli*, ETEC)**	NCCP	ND	*Vibrio parahaemolyticus*	KCCM	ND
***Epstein-Barr* virus**	ZMC	ND	*Vibrio vulnificus*	KCCM	ND
Giardia intestinalis	ATCC	ND	*Yersinia enterocolitica*	KCCM	ND

Abbreviations: ATCC, American type culture collection; BEI resources, the biological and emerging infections resources program; KCCM, Korean culture center of microorganisms; KCTC, Korean collection for type culture; NCCP, national culture collection for pathogens; ND, not detected; ZMC, ZeptoMerix.

strated that an assay that can simultaneously detect six different types of viruses in a single tube, using the MuDT technique, can potentially be the most useful technique for the detection of the gastroenteritis-causing pathogens.

This study has several limitations. Clinical samples that showed inconsistent results after the application of both assays were additionally sequenced, and this demonstrated that 18 samples that were determined to be positive by the Allplex GI-virus Assay were true positives, whereas nine samples determined to be positive by the Seeplex Diarrhea-V ACE Detection were not analyzed. Additionally, the Allplex GI-virus Assay detected 19 SV-positive samples among 1489 stool specimens, but this does not reflect the total amount of SV-positive samples. Therefore, these samples should be compared further to commercial multiplex assays that can detect identical viral targets, such as FilmArray gastrointestinal panel (BioFire, Salt Lake City, UT). SV is a crucial causative pathogen leading to the development of sporadic gastroenteritis in children. Therefore, although the prevalence of this type of gastroenteritis is not high, the detection of this difficult-to-culture virus is very important (24), and it is necessary to evaluate the performance of multiplex assays for detection of SV.

In conclusion, even though it is difficult to completely confirm the advantages of the Allplex GI-virus Assay over currently existing methods in the detection of viral gastroenteritis-causing agents, additional validation of its ability to detect SV may potentially lead to the replacement of conventional PCR assays used for molecular diagnosis.

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Footnotes

Author Contribution: Study concept and design: Jeong-Hyun Han; acquisition of data: Jeong-Hyun Han and Sun-Hyung Kim; statistical analysis: Jeong-Hyun Han and Sun-Hyung Kim; drafting of the manuscript: Jeong-Hyun Han; administrative, technical and material support: Jeong-Hyun Han and Sun-Hyung Kim; critical revision of the

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Table 3. Repeatability of the Allplex-GI virus Assay

Sample		Within-Run (n = 80)		Run-to-Run (n = 40)		Day to Day (n = 20)	
		Mean ± SD	CV (%)	Mean ± SD	CV (%)	Mean ± SD	CV (%)
NVGI	Moderately positive	34.10 ± 0.97	2.85	34.10 ± 0.93	2.72	34.10 ± 0.76	2.24
	Minimally positive	36.58 ± 1.71	4.66	36.59 ± 1.47	4.01	36.56 ± 1.05	2.89
	Highly negative	38.47 ± 1.76	4.56	38.47 ± 1.67	4.33	38.24 ± 0.93	2.44
NVGII	Moderately positive	34.98 ± 0.75	2.15	34.98 ± 0.71	2.03	34.98 ± 0.66	1.89
	Minimally positive	37.25 ± 1.01	2.71	37.25 ± 0.86	2.32	37.26 ± 0.73	1.97
	Highly negative	39.03 ± 1.25	3.19	39.07 ± 1.12	2.87	39.06 ± 1.03	2.64
ADV-F	Moderately positive	33.60 ± 0.92	2.74	33.60 ± 0.89	2.64	33.60 ± 0.70	2.09
	Minimally positive	34.85 ± 0.89	2.56	34.85 ± 0.78	2.23	34.85 ± 0.57	1.64
	Highly negative	36.92 ± 1.24	3.36	36.88 ± 0.95	2.58	36.93 ± 0.49	1.33
ROV	Moderately positive	35.19 ± 1.01	2.87	35.19 ± 0.84	2.38	35.19 ± 0.59	1.69
	Minimally positive	37.02 ± 1.03	2.78	37.02 ± 0.94	2.53	37.02 ± 0.65	1.75
	Highly negative	38.43 ± 1.02	2.67	38.52 ± 0.80	2.09	38.51 ± 0.63	1.64
ASV	Moderately positive	34.95 ± 0.69	1.98	34.95 ± 0.66	1.89	34.95 ± 0.41	1.17
	Minimally positive	37.58 ± 1.24	3.30	37.58 ± 1.20	3.20	37.58 ± 0.92	2.46
	Highly negative	40.07 ± 0.84	2.09	40.04 ± 0.80	1.99	40.07 ± 0.37	0.93
SV	Moderately positive	33.81 ± 0.66	1.94	33.81 ± 0.59	1.74	33.81 ± 0.50	1.47
	Minimally positive	36.70 ± 0.99	2.70	36.70 ± 0.95	2.60	36.70 ± 0.77	2.09
	Highly negative	38.95 ± 0.96	2.47	38.95 ± 0.90	2.31	38.95 ± 0.78	2.00

Abbreviations: NVGI, norovirus GI; NVGII, norovirus GII; ADV-F, adenovirus F; ROV, rotavirus; ASV, astrovirus; SV, sapovirus; SD, standard deviation; CV, coefficient of variation.

Table 4. Performance of Allplex GI-virus Assay in Comparison with Seeplex Diarrhea-V ACE Detection

Virus	No. of Positive Samples		No. of Negative Samples		Agreement				Cohen's Kappa	
					Positive		Negative			
	Allplex	Seeplex	Allplex	Seeplex	%	95% CI	%	95% CI	%	95% CI
Total	296	290	7149	7155	94.9	93.1 - 96.7	98.8	98.3 - 99.2	0.95	0.92 - 0.96
NVGI	20	18	1469	1471	89.5	79.2 - 99.7	99.9	99.7 - 100	0.89	0.71 - 0.94
NVGII	110	112	1379	1377	96.4	93.9 - 98.9	99.7	99.5 - 99.9	0.96	0.92 - 0.98
ADV-F	23	20	1466	1469	88.4	78.2 - 98.5	99.8	99.7 - 100	0.88	0.72 - 0.93
ROV	118	113	1371	1376	95.2	92.4 - 98.0	99.6	99.4 - 99.8	0.95	0.90 - 0.97
ASV	25	27	1464	1462	96.2	90.8 - 100	99.9	99.8 - 100	0.96	0.84 - 0.96

Abbreviations: Allplex, Allplex GI-virus assay; Seeplex, Seeplex diarrhea-V ACE detection.

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Table 5. Analysis of Specimens Where the Discrepant Results were Obtained Using Allplex GI-Virus Assay and Seeplex Diarrhea-V ACE Detection

Virus	Obtained Results			No. of Positive Sequencing Results
	Allplex	Seeplex	No. of Specimens	
NVGI	Positive	Negative	3	3
	Negative	Positive	1	0
NVGII	Positive	Negative	3	3
	Negative	Positive	5	2
ADV-F	Positive	Negative	4	4
	Negative	Positive	1	0
ROV	Positive	Negative	8	8
	Negative	Positive	0	0
ASV	Negative	Positive	2	1

Abbreviations: Allplex, Allplex GI-virus assay; Seeplex, Seeplex diarrhea-V ACE detection.

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