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Evaluation of the NucliSens® Basic Kit assay for detection of Norwalk virus RNA in stool specimens

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Abstract

Norwalk-like viruses (NLVs) are a genetically diverse group of human caliciviruses that are the most common cause of epidemic gastroenteritis and are detected typically in stool by reverse transcription (RT)-PCR or electron microscopy (EM). The application of a rapid nucleic acid sequence-based amplification (NASBA) assay for the detection of NLV RNA in stool is described using the NucliSens® Basic Kit. Primers and probes for the NLV Basic Kit assay were based on the RNA polymerase region of the prototype NLV, Norwalk virus (NV) genome and could consistently detect 10^4 RT-PCR detectable units of NV RNA in a stool filtrate. When compared directly with RT-PCR on a dilution series of NV stool filtrate, the NucliSens® Basic Kit assay was equally sensitive. Cross-reactivity studies with a representative panel of other enteric pathogens were negative. When applied to 15 stool specimens from NV-challenged volunteers, the NASBA Basic Kit application for NV detection yielded 100% sensitivity, 50% specificity, and 67% concordance, using RT-PCR as the 'gold standard'. Despite the specificity of the NASBA primer/probe sequences for NV, other representatives from both NLV genogroups I and II could be detected by the Basic Kit assay in outbreak stool specimens, although the results were inconsistent. Our results suggest that the NucliSens® Basic Kit assay provides a rapid and sensitive alternative to RT-PCR for detecting NV RNA in stool specimens. However, improvements in test specificity and primer design will be needed before the assay can be used routinely in the clinical setting.

Keywords: Norwalk virus; Norovirus; NASBA; NucliSens®; Basic Kit; Viral gastroenteritis; Stool

1. Introduction

Norwalk-like viruses (NLVs), also called '*Noroviruses*', is a genetically diverse genus that is classified in the family *Caliciviridae* (Green et al., 2000). These viruses contain a positive-sense, single stranded RNA genome of approximately 7.6 kb. Based on sequence information for the genes encoding the viral RNA-dependent RNA polymerase and the capsid protein, the NLV genus is sub-divided further into two genogroups: Genogroup I with Norwalk virus (NV) as the prototype strain, and Genogroup II with Snow Mountain and Hawaii virus as prototype strains. NLVs have been implicated in 96% of the outbreaks of acute nonbacterial gastroenteritis in the US documented by the Centers for Disease Control and Prevention (CDC) between 1996 and 1997 (Fankhauser et al., 1998). Additionally, NLVs are a major cause of acute nonbacterial gastroenteritis in children and adults worldwide (Bon et al., 1999; Parks et al., 1999; Taylor et al., 1996; Vinje et al., 1997). NLV outbreaks have been reported in communities, schools and day care centers, nursing homes, hospitals, recreational areas, cruise ships, military ships and bases

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(Fankhauser et al., 1998; Grohmann et al., 1991; McCarthy et al., 2000; Sharp et al., 1995). These viruses replicate in the gastrointestinal tract of the infected host and are excreted in feces. Although food-borne transmission is most common, these viruses are also transmitted through fecal contamination of drinking and recreational water, as well as through contact with contaminated environmental surfaces and infected individuals.

Despite numerous attempts, NLV infections have not been induced in experimental animals, nor have these viruses been propagated successfully in cell culture. Diagnosis has relied historically on the observation of the 27 nm viral particles by electron microscopy (EM) (Glass et al., 2000). However, because NLVs are shed usually in low concentrations, EM is frequently not sensitive enough to detect these viruses in stool. Early diagnostic tests for NV, developed in the 1970's and 1980's, were primarily immunoassays that required reagents derived from NV-infected human volunteers and were not widely available (Atmar and Estes, 2001). The cloning and sequencing of NV in 1990 (Jiang et al., 1990) led to the development of new molecular diagnostic assays. Currently, the detection method used most widely for NLVs in stool samples is reverse transcription (RT)-PCR. The resulting RT-PCR amplicons are confirmed by probe hybridization or nucleotide sequence analysis. RT-PCR has proven to be an extremely sensitive method for detection of NLVs. However, this method has several drawbacks: the presence of amplification inhibitors in stool samples, the need to confirm amplification products to prevent false positive results, and the amount of time needed to complete the analysis.

Rapid identification of pathogens is important for patient care and for prompt, appropriate interventions to control outbreaks of disease. Studies have demonstrated that the isothermal nucleic acid sequence-based amplification (NASBA) method provides a sensitive and rapid method for detection of viral RNA from human immunodeficiency virus type 1 (HIV-1) (Berndt et al., 2000; Fiscus et al., 2000; Segondy et al., 1998; van Gemen et al., 1993), Epstein-Barr virus (EBV) (Brink et al., 1998; Cruz et al., 2000), human cytomegalovirus (HCMV) (Blok et al., 2000; Witt et al., 2000), human rhinovirus and enterovirus (Damen et al., 1999; Lunel et al., 1999). This RNA amplification method utilizes three enzymes (reverse transcriptase, T7 RNA polymerase, and RNaseH) and two target specific oligonucleotide primers. The reverse oligonucleotide primer contains the bacteriophage T7 promoter sequence, while the forward oligonucleotide primer contains an electrochemiluminescent (ECL) tail that is used in the detection of the amplification product. The transcription-driven NASBA reaction amplifies theoretically the RNA target more than 10^{12}-fold within 90 min. In this study, we report the application of a rapid NASBA assay for the detection of NV RNA in stool using the NucliSens® Basic Kit. This assay was compared directly with RT-PCR and used for the detection of NV and other NLVs in fecal specimens obtained from human challenge studies and NLV outbreaks. The NASBA assay described here was optimized for the detection of NV, and these NASBA primers and probes were not designed to be reactive broadly for other NLV.

2. Materials and methods

2.1. Virus preparations for assay evaluation

The NV stock preparation used in this study was made from a 20% suspension in PBS of a liquid stool from a volunteer infected with the NV 8FIIa inoculum (Dolin et al., 1971). The stool suspension was trichlorotrifluoroethane (Freon)-extracted three times then filtered through a 0.2 μm filter. The first and second filtrates were pooled, aliquoted into 1 ml portions, and stored at −70 °C. Virus concentration was estimated by endpoint titration RT-PCR using the heat release method (Schwab et al., 1997) and oligonucleotide primers based on the sequence of the NV polymerase gene (NV51/NV3) (Moe et al., 1994). Estimates of NV concentration in the stock preparation ranged from 4×10^6 to 6×10^7 PCR detectable units (PDU) per ml with a mean of 2×10^7 PDU/ml.

2.2. Viruses and bacterial strains used for evaluating assay specificity

Poliovirus type 1 (PV1), strain LSc, was propagated in BGMK (African green-monkey kidney derived) cells and titered using the plaque technique (Sobsey, 1987). Hepatitis A virus (HAV, cytopathic strain HM-175) was grown and plaque assayed for enumeration using FRhK-4 (fetal rhesus monkey kidney-derived) cells (Cromeans et al., 1987). Other viruses in the test panel included adenovirus (Ad5), astrovirus (Ast5), human rotavirus (HRV305) (gifts from Mark Sobsey, Department of Environmental Science and Engineering, UNC-CH) and coronavirus (MHV-A59) (gift from Ralph Baric, Department of Epidemiology, UNC-CH). Gram negative bacterial strains, including *Escherichia coli* ATCC 25922 and *E. coli* O157: H7 (HC 122), were cultivated overnight at 35 °C in brain heart infusion (BHI) broth (Difco, Detroit, MI). *Clostridium perfringens* (NCTC 8798) was cultivated in fluid thioglycollate (FTG) medium (Difco) for 24 h at 37 °C.

2.3. Volunteer and outbreak stool specimens for assay evaluation

Aliquots of stool specimens collected from a NV human challenge study (Moe et al., 1998) were prepared as 10–20% suspensions in sterile water and stored at 4 °C prior to use. Fecal specimens collected from several NLV outbreaks were prepared in a similar manner.

2.4. Viral RNA isolation from the NV stock preparation and stool specimens

2.4.1. Viral RNA isolation for RT-PCR assay

Stool specimens from the NV human challenge study and the NLV outbreaks were tested by RT-PCR using a modified heat release method (Schwab et al., 1997) or the Ultraspec3 (Biotecx, Houston, TX) RNA extraction method according to the manufacturer's directions.

2.4.2. Viral RNA isolation for the Basic Kit Assay

NV stock preparation containing 2×10^7 PDU/ml was serially diluted 10-fold in the NucliSens® Basic Kit Lysis Buffer (bioMérieux Inc., Durham, NC), and the RNA was extracted as per manufacturer's instructions (extraction method based on that of Boom et al., 1990). Briefly, the NV stock-lysis buffer mixture was mixed with activated silica, the bound viral nucleic acid was washed once with 1 ml wash buffer [10M GuSCU, 100mM Tris–HCl (ph 6.4)], twice with 1 ml 70% ethanol, and once with 1 ml acetone. After each wash step, the suspension was briefly centrifuged, and the silica pellet was suspended in the next wash solution. Following a drying step at 56 °C for 10 min, the RNA was eluted from the silica by incubation in 50 μl of NucliSens® Basic Kit (bioMérieux) elution buffer for 10 min at 56 °C. Purified RNA was stored at −80 °C prior to use in amplification reactions. For the outbreak and challenge study stool specimens, a 10% stool suspension (rather than 20%) was prepared in sterile nuclease-free water and the samples were pre-treated for removal of inhibitors by centrifugation at $2000 \times g$ for 15 min. An additional ethanol wash was incorporated after the buffer wash step to facilitate removal of residual amplification inhibitors.

2.5. RT-PCR and southern hybridization

RT-PCR on a dilution series of NV stock preparation was carried out using 5 μl of the purified viral RNA and the oligonucleotide primer pair NV3/NV51, that produces a 206-bp amplicon corresponding to nucleotides 4673–4878 within the RNA polymerase gene of NV (Moe et al., 1994). Reactions were carried out with the RT-PCR Access System kit (Promega Corporation, Madison, WI) according to manufacturer's protocol and as previously reported (Moe et al., 1994). The stool specimens from the human challenge studies and the NLV outbreaks were tested by RT-PCR using the G1 (SR33/SR48) and G2 primers, that produce a 123-bp product between nucleotides 4754–4876, also within the RNA polymerase gene (Ando et al., 1995). The G1 and G2 primers are more broadly reactive than the NV3/NV51 pair and were routinely used to screen volunteer and outbreak specimens for the presence of any NLV infection. Oligonucleotide primers were prepared by the Pathology Oligonucleotide Facility at the University of North Carolina at Chapel Hill. Briefly, RT was done at 48 °C for 60 min. After heat inactivation of the reverse transcriptase at 94 °C for 3 min, the samples were subjected to 40 cycles of 94 °C for 1 min, 42 °C for 1.0 min 20 s, and 72 °C for 1 min followed by a final extension at 72 °C for 15 min. The resulting DNA amplicons were electrophoresed on a 3% agarose gel and stained with ethidium bromide. Electrophoresed RT-PCR amplicons were transferred to Hybond-N^+ (Pharmacia Amersham Biotech, Piscastaway, NJ). Hybridization was performed at 41 °C with the fluorescein-11-dUTP labeled (3′ Oligo Labeling and Detection kit, Pharmacia Amersham Biotech) using the NVInt oligonucleotide probe. RT-PCR amplicons were sequenced as described previously (Moe et al., 2001). Primer and probe sequences are listed in Table 1.

2.6. NASBA amplification and ECL detection

NASBA oligonucleotide primers (BKP 1.2/BKP 2.2) corresponded to the NV3/NV51 pair and amplified a 206 bp region of the NV RNA polymerase gene (Accession number 86771). These primers were designed and labeled with the T7 RNA polymerase promoter and ECL detection sequences, respectively, through cooperation with bioMérieux (Table 1). NASBA reactions used 5 μl of purified viral RNA, a 10 μM primer concentration, and 70 mM KCl, with reactions performed according to the NucliSens® Basic Kit instructions. Briefly, 10 μl of the premix cocktail was dispensed into thin-walled Eppendorf tubes and 5 μl of nucleic acid eluate was added to each tube. The tubes were incubated at 65 °C for 5 min followed by cooling to 41 °C for 5 min. A 5-μl aliquot of enzyme mix (RNA polymerase, RNase H, and AMV-reverse transcriptase) was added to each tube followed by incubation for 90 min at 41 °C (in water bath or thermocycler). ECL detection was done immediately after amplification; alternatively, the amplified RNA was stored at −20 °C (for no more than 7 days) prior to ECL detection.

The NASBA-generated products were detected by liquid hybridization using the electrochemiluminescence (ECL) principle employed by the NucliSens® reader. This method uses two oligonucleotides i.e. (i) a specific capture oligonucleotide immobilized onto paramagnetic

Table 1
Sequences of oligonucleotide primers and probes used for RT-PCR and NucliSens® Basic Kit detection of NV RNA

Primer	Nucleotide sequence[a]	Polarity	Application
BKP 1.2	5′-aattctaatacgactcactatagggagaaggGTTGACACAATCTCATCATCA-3′	(−)	NASBA
BKP 2.2	5′-gatgcaaggtcgcatatgaggCACCATCTGAGATGGATGTA-3′	(+)	NASBA
SR33	5′-TGTCACGATCTCATCATCACC-3′	(−)	RT-PCR
SR48	5′-GTGAACAGCATAAATCACTGG-3′	(+)	RT-PCR
NV3	5′-GCACCATCTGAGATGGATGT-3′	(+)	RT-PCR
NV51	5′-GTTGACACAATCTCATCATC-3′	(−)	RT-PCR
NVINT	5′-ACTGGTTTATCACCTGATGTG-3′[b]	(−)	Hybridization(Southern and ECL)

[a] Uppercase letters designate genome sequences used for amplification or hybridization as designated. Lowercase letters indicate T7 promoter sequence (BKP 1.2) and the sequence specific for the NucliSens® ruthenium-linked oligonucleotide detector probe (BKP2.2).
[b] NVInt was 5′ biotinylated during its manufacture to facilitate binding to streptavidin-labeled magnetic spheres for capture of specific amplicons and subsequent detection using the NucliSens® ECL Detection module.

beads; and (ii) a second detector oligonucleotide complexed to a ruthenium chelate. The specific capture probe used in this assay corresponded to the same NVInt probe used in RT-PCR hybridizations, except that it was 5′ biotinylated during manufacture to facilitate its complex to streptavidin-coated magnetic beads. The detector probe sequence is generic, provided by the NucliSens® Basic Kit, and complementary to the 3′ terminal end of the amplicons which are transcribed from the stretch of nucleotides identified for primer BKP 2.2 (see Table 1). Amplification products (5 μl) were diluted in 100 μl of detection buffer provided with the NucliSens® Basic kit. Hybridization was carried out using 20 μl of a 1:1 mixture of streptavidin-coated capture probes coupled to magnetic beads and Ru^{2+}-labeled oligonucleotide detection probes, and 5 μl of the 1:20 diluted amplification product. The solution was incubated at 41 °C for 30 min after which assay buffer (300 μl) was added to the tubes followed by detection using the NucliSens® ECL reader (bioMérieux).

After incubation, the paramagnetic beads carrying the hybridized amplicon/ECL probe complexes were magnetically captured on the surface of an electrode that is part of the ECL reader. Voltage applied to the electrode triggers the ECL reaction, such that the light emitted by the hybridized Ru^{2+} -labeled probes is proportional to the quantity of amplicon present. The ECL reader detects this luminescent energy and converts it to a digital read-out. The reader further assigns each specimen a positive or negative outcome based on the luminescent signal of the specimen relative to the positive and negative sample controls, and performance controls provided by the manufacturer. ECL results were transformed to $\log_{10}$ values for subsequent analyses using MICROSOFT EXCEL (Redmond City, WA).

2.7. Sequence analysis of NASBA generated amplicon

RT-PCR was done using 1 μl of NASBA generated amplification product and the oligonucleotide primer pair NV3/NV51 (Moe et al., 1994). Reactions were performed as described above. The resulting DNA amplicons were electrophoresed on a 3% agarose gel and visualized with ethidium bromide. For sequence analysis, the resulting 206 bp RT-PCR products were purified (Qiagen PCR, Qiagen Valencia CA) and the nucleotide sequences were determined at the University of North Carolina at Chapel Hill Automated Sequencing Facility using primers NV3 and NV51. Nucleotide sequence analyses were done using the Wisconsin Genetic Computer Group (GCG) software and Basic Local Alignment Search Tool (BLAST) analysis (Altschul et al., 1990).

3. Results

3.1. Detection limits and reproducibility of the NucliSens® Basic Kit assay for the detection of NV RNA

To evaluate the limit of detection and the reproducibility of the Basic Kit assay for detection of NV RNA, serial 10-fold dilutions were made of the NV stock preparation (corresponding to 10^{-4} to 10^{5} PDU/ml) and the entire Basic Kit method was applied from RNA isolation through NASBA amplification and ECL detection. Each dilution was tested in triplicate on three different days, resulting in nine replicate tests per dilution. Consistent detection of NV RNA was possible for dilutions corresponding to 10^{4} PDU/ml with very small relative standard deviations (0.1–0.6). As the concentration of NV was reduced to the equivalent of 10^{2} PDU/ml, NASBA detection was slightly less consistent, with eight of the nine reactions giving positive amplification results. At this dilution, larger standard deviations were observed only in those instances where one of three samples in a run was negative for viral RNA. The detection endpoint occurred at approximately 10^{1} PDU/ml. At levels of 10^{0} PDU/ml and below, the NASBA results were consistently negative and had small standard deviations. These results are summarized in Fig. 1, which shows fairly reliable

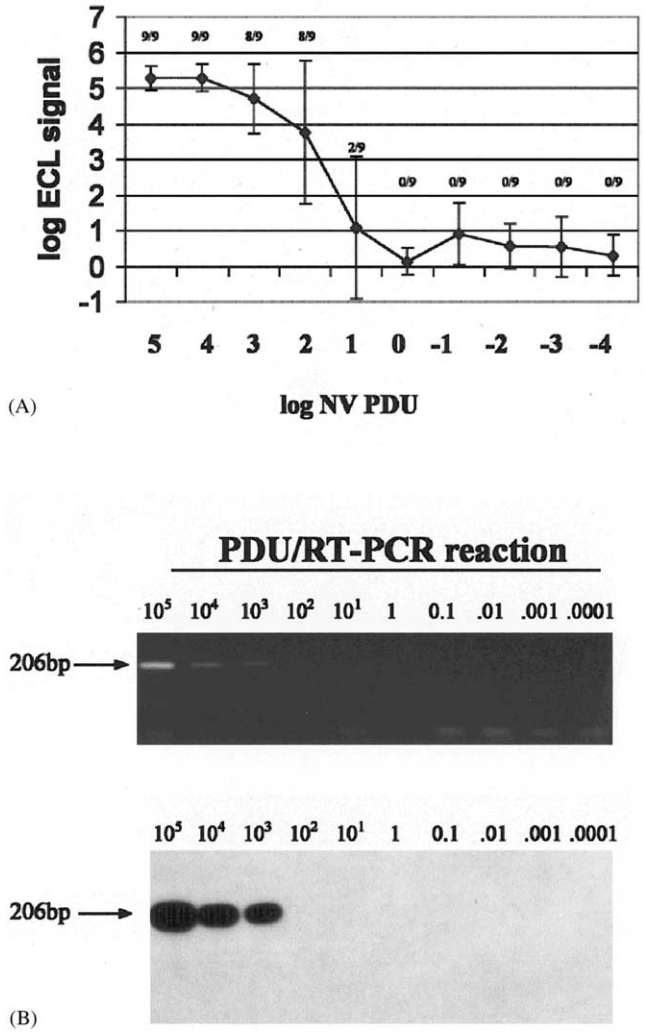

Fig. 1. (A). Detection limits and reproducibility of the NucliSens® Basic Kit for the detection of NV RNA. Results are displayed as mean ± standard deviation (S.D.) $\log_{10}$ ECL readings of triplicate samples from three replicate assays (i.e. nine samples). (B). Representative detection limits for RT-PCR and Southern hybridization analysis using the same NucliSens® Basic Kit RNA extracts used in (A) above.

detection of NV RNA by the NucliSens® Basic Kit assay at levels of 10^5–10^2 PDU/ml from serially-diluted 20% stool suspensions. When the same RNA preparations were tested by RT-PCR using the NV3/NV51 primer set and followed by Southern hybridization, we observed that the NASBA amplification method provided 10–100-fold more sensitive detection limits. Furthermore, the NucliSens® Basic Kit assay was completed in 4–6 h compared with 48 h for RT-PCR with Southern hybridization.

3.2. Cross-reactivity of the NucliSens® Basic Kit assay

To ascertain whether the BKP 1.2/BKP 2.2 amplification primers and the NVInt probe were specific for NV amplification and detection, this primer/probe combination was evaluated with a panel of representative bacterial and viral enteric pathogens (Table 2). ECL detection was negative for the entire panel, with mean $\log_{10}$ ECL signals of ≤ 1.6 as compared with the mean $\log_{10}$ ECL value of 4.5 for the NV-positive specimen.

Table 2
Cross-reactivity of NucliSens® Basic kit using BKP 1.2/BKP 2.2 amplification primers and NVInt hybridization probe for ECL detection against selected enteric microorganisms

Organism	Mean $\log_{10}$ ECL signal[a]	Interpretation
NV	4.5	(+)
Adenovirus 5	0.6	(−)
Astrovirus 5	0	(−)
Coronavirus	0	(−)
HAV-HM175	0	(−)
Poliovirus type 1	0	(−)
Human rotavirus	1.6	(−)
Escherichia coli	0	(−)
E. coli O157: H7	0	(−)
Clostridium perfringens	0	(−)

[a] $\log_{10}$ ECL readings reflect mean of 2–5 duplicate samples.

3.3. Comparative sensitivity of the NucliSens® Basic Kit method to RT-PCR

To compare the clinical sensitivity of the NucliSens® Basic Kit assay to RT-PCR for the detection of NV RNA in stool specimens, 15 stool specimens from infected and uninfected volunteers in an NV challenge study collected at pre-challenge and various times post-challenge were tested by RT-PCR and NASBA (Table 3). NV infection in these volunteers was also confirmed by ELISA for IgG seroconversion as described previously (Moe et al., 1998). All five specimens that were positive by RT-PCR were confirmed by a positive ECL signal, yielding a test sensitivity of 100% when using RT-PCR as the 'gold standard'. The $\log_{10}$ of these positive ECL values ranged from 4.82 to 5.56 with a geometric mean of 5.14. Five specimens that were negative by RT-PCR were also confirmed as negative by the NucliSens® Basic Kit assay. However, there were two volunteer samples (VT 23-1, VT 5-1) that were negative by RT-PCR but gave inconsistently positive results using the NucliSens® Basic Kit. These specimens tested negative in one assay but positive in another and can be considered true false positives because they were obtained from subjects prior to NV challenge. There also were three RT-PCR negative samples that gave consistently positive results by NASBA-ECL. The $\log_{10}$ ECL signals for these samples ranged from 3.48 to 4.3, with a geometric mean of 3.97 that was more than one $\log_{10}$ lower than the geometric mean of the RT-PCR positive/NucliSens® Basic Kit positive samples (5.14). Sequence analysis of the NASBA amplicons obtained from these three samples confirmed the pre-

Table 3
Comparative sensitivity of the NucliSens® Basic Kit assay to RT-PCR for detection of NV RNA in representative stool specimens collected from NV-challenged volunteers

Sample	IgG seroconversion[a]	Day post-challenge[b]	RT-PCR[c]	NucliSens® Basic Kit Assay[d]
VT 3-1	N	0	(−)	(−) (0.87)
VT 5-1	N	0	(−)	(+/−) (2.39) [3.93, 0.85]
VT 23-1	Y	0	(−)	(+/−) (3.53) [4.38, 4.65, 1.59]
VT 44-1	Y	0	(−)	(−) (0.48)
VT 3-5	N	4	(−)	(+) (4.13)
VT 3-8	N	6	(−)	(−) (0)
VT 5-5	N	4	(−)	(−) (0)
VT 5-8	N	8	(−)	(−) (0)
VT 23-3	Y	2	(+)	(+) (5.32)
VT 23-4	Y	3	(+)	(+) (5.56)
VT 23-8	Y	22	(−)	(+) (4.30)
VT 44-5	Y	9	(+)	(+) (4.99)
VT 44-6	Y	16	(+)	(+) (4.82)
VT 44-7	Y	23	(−)	(+) (3.48)
VT-P	NA	NA	(+)	(+) (4.99)

[a] NV infection status based on IgG seroconversion in volunteer.
[b] Day post-challenge that stool sample was collected. Zero, (0), baseline sample collected before NV challenge.
[c] (+/−) by RT-PCR using the SR33/SR48 primer pair; RT-PCR amplicon detection by agarose gel electrophoresis and Southern hybridization.
[d] (+/−) by NucliSens® Basic Kit assay; second set of parenthetical results indicate mean $\log_{10}$ ECL signal; third set of parenthetical results indicate the individual $\log_{10}$ ECL signals from replicate tests on specimens with ambiguous (+/−) results.

sence of the target NV RNA. Two of these three specimens (VT 23-8 and VT44-7) were collected from NV-infected volunteers at later post-challenge times (22–23 days post-challenge) than the other RT-PCR positive specimens from these volunteers (2–16 days post-challenge). We have detected NV-positive specimens at Day 22 post-challenge by RT-PCR in only two other infected volunteers (data not shown) indicating that viral shedding is possible during this time period. However, those specimens were not tested by the NucliSens® Basic Kit. The NASBA results for VT 23-8 and VT 44-7 suggest that the NucliSens® Basic Kit may be more sensitive than the RT-PCR assay. However, this statement is made cautiously because a third RT-PCR negative specimen (VT 3-5 from Day 4 post-challenge) that was positive by the NucliSens® Basic Kit assay was from a volunteer who had no indication of viral shedding by RT-PCR and who did not seroconvert. Overall specificity (using the RT-PCR gold standard) was 50% and concordance between the RT-PCR and NASBA results was 67%.

3.4. Comparative specificity of the NucliSens® Basic Kit NASBA method to RT-PCR for outbreak stool specimens

To examine the ability of the Basic Kit assay to detect other NLVs using the oligonucleotide primer/probe set based on the NV genome, six RT-PCR positive stool specimens (CDC 350 (GI), DF-1 (GI), FS-1 (GI), LV-1 (GII), MC-1 (GII), and Toronto (GII)) from NLV outbreaks were tested using the NucliSens® Basic Kit assay described above. Only one of the three Genogroup I outbreak strains (CDC 350 with 78% nucleotide homology to NV) was positive by the NASBA/ECL assay, although the degree of positivity was low and not consistent from run to run. The other two strains with 69% homology to NV were negative (DF-1, FS-1). Of the Genogroup II outbreak strains (LV-1, MC-1 and Toronto, with 60–63% homology to NV), the Toronto strain tested positive using the NucliSens® Basic Kit assay and NV-based primers and detection probes developed in this study. However, as seen for GI strain CDC 350, the degree of positivity was low and not consistent from run to run. The other two GII strains were consistently negative (data not shown).

4. Discussion

In this study, we report the application of a nucleic acid sequence based amplification assay (NASBA) for the detection of the NLV prototype NV 8FIIa strain using the NucliSens® Basic Kit. The NucliSens® Basic Kit assay compared favorably to parallel RT-PCR assays with respect to assay detection limits. However, the NucliSens® Basic Kit assay provides a more rapid alternative to RT-PCR, with completion in 4–6 h with the former as compared with 48 h with the latter. Furthermore, the kit format for the Boom et al. (1990)RNA extraction method provided ease and standardization for less experienced laboratory personnel. The isothermal amplification approach used by the Basic Kit also simplifies equipment needs and costs compared with the use of thermocycling devices. In

short, the NucliSens® Basic Kit provides a promising nucleic acid amplification alternative that combines amplification, detection and confirmation into a simplified and streamlined protocol.

NASBA has proven to be a sensitive and specific assay for use in clinical diagnostic microbiology, with commercial kits available for the detection of human HIV, EBV and CMV in blood and plasma, many of which are quantitative assays. Investigators have reported particularly good success using NASBA methods to quantitate HIV load in blood and plasma (Berndt et al., 2000; Dyer et al., 1999). More recently, the technology has been developed for the detection and/or quantification of HCV (Damen et al., 1999), *Candida* species (Widjojoatmodjo et al., 1999) and the parasite *Plasmodium falciparum* (Schoone et al., 2000), all in blood-based specimens. RNA sequences from *Chlamydia trachomatis* derived from urine and cervical scrapings (Morre et al., 1996), as well as rhinovirus in nasopharangeal aspirates (Samuelson et al., 1998) have also been detected using NASBA approaches. To our knowledge, this is the first published report on the use of NASBA technology for the detection of an enteric pathogen in a fecal matrix. Overall, the NucliSens® Basic Kit method for RNA extraction resulted in high-quality RNA extracts that were capable of supporting NASBA amplification with no further sample dilution. Because levels of infectious virus in patients during acute phases of viral gastroenteritis can approach 10^6–10^8/g of feces (Kapikian et al., 1996), the potential of this method for routine diagnosis of NV infection is excellent.

As with RT-PCR assays, the identification of broadly reactive primers for the detection of human caliciviruses remains a challenge. The primers and probe set used in this study were intended to demonstrate the feasibility of NASBA amplification for the detection of Norwalk virus in stool, and were, therefore, designed to be specific for the G1 NV 8FIIa strain. Although we were readily able to detect the prototype Norwalk virus strain 8FIIa with these primers, they were relatively ineffective for the detection of other Genogroup I and II NLV strains collected from food-borne disease outbreaks. Even though these results were anticipated, this finding further supports the need for more broadly reactive primers to improve all nucleic acid amplification assays designed for the detection of this genetically diverse group of viral pathogens.

Different RNA extraction methods were used for the NASBA assay and the RT-PCR assay. For the NASBA assay, the NucliSens® Basic Kit RNA extraction method, based on the method of Boom et al. (1990), was used as per the manufacturer's instructions. For the RT-PCR assay, we used two methods that work well in our experience: the heat release method and the Ultraspec3 method. These different methods may explain in part the discrepant results from the two assays for a few specimens. It is well known that the sensitivity and specificity of nucleic acid amplification methods is affected by the efficiency of the RNA isolation method. For instance, Brink et al. (Brink et al., 1998) noted that the relative sensitivity of NASBA detection of EBV is more likely to be influenced by RNA isolation efficiency than by amplification efficiency. Others have shown that the effect of matrix-associated inhibitors on amplification efficiency is exacerbated at low levels of target template, yielding both false negative and false positive results (Jaykus et al., 1996). Losses during RNA extraction may well account for the slightly poorer detection limits noted for both the RT-PCR and the NucliSens® Basic Kit methods described in this paper (Fig. 1), when compared with the RT-PCR detection limits we have observed for dilutions of the Norwalk virus stock preparation. The Norwalk virus stock solutions used in this study were titered using a heat-release RT-PCR method (Schwab et al., 1997) rather than the Boom et al. (1990) silica-based method followed by RT-PCR. The relatively high degree of false positive amplification results noted in the Basic Kit assay may also be associated with residual matrix components that serve as template for non-specific amplification and hybridization. It may be possible to improve both the sensitivity and specificity of amplification by using an alternative RNA extraction method, followed by amplification and detection using the latter two steps of the NucliSens® Basic Kit. Such an approach could facilitate further removal of residual matrix associated components and thereby improve the efficiency and specificity of subsequent RNA amplification.

This study demonstrates the use of the NucliSens® Basic Kit in the application of a NASBA/ECL method for the rapid and sensitive detection of NV RNA in fecal samples. To our knowledge, this is the first study that has sought to apply NASBA/ECL amplification and detection technology to the group of NLV. Furthermore, because the virus stock used in these experiments had been titered previously by heat-release RT-PCR multiple times, we have been able to establish an approximate level of detection when applying the combined RNA extraction, NASBA amplification, and ECL detection of the NucliSens® Basic Kit. The detection limit approximates 10^1–10^3 PDU per amplification reaction, which is very close to the detection limits obtained for RT-PCR. However, the ease of use and speed of detection supplied by the NucliSens® system provides a significant advantage over RT-PCR detection approaches. With further development and optimization, particularly with respect to primer design and assay specificity, we are confident that NASBA/ECL technology will continue to develop as a viable method to detect enteric pathogens in fecal matrices.

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