

ORIGINAL ARTICLE

A nucleic acid sequence-based amplification assay for real-time detection of norovirus genogroup II

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Abstract

Aims: To use molecular beacon based nucleic acid sequence-based amplification (NASBA) to develop a rapid, sensitive, specific detection method for norovirus (NV) genogroupII (GII).

Methods and Results: A method to detect NV GII from environmental samples using real-time NASBA was developed. This method was routinely sensitive to 100 copies of target RNA and intermittent amplification occurred with as few as 10 copies. Quantitative estimates of viral load were possible over at least four orders of magnitude.

Conclusions: The NASBA method described here is a reliable and sensitive assay for the detection of NV. This method has the potential to be linked to a handheld NASBA device that would make this real-time assay a portable and inexpensive alternative to bench-top, lab-based assays.

Significance and Impact of the Study: The development of the real-time NASBA assay described here has resulted in a simple, rapid (<1 h), convenient testing format for NV. To our knowledge, this is the first example of a molecular beacon based NASBA assay for NV.

Introduction

Noroviruses (NV) are a group of small nonenveloped RNA viruses that belong to the Caliciviridae family. NVs cause epidemic acute gastrointestinal illness in humans and are thought to be the most common cause of viral gastroenteritis worldwide (Fankhauser *et al.* 1998; Lopman *et al.* 2003). NV strains causing human infections can be subdivided into at least two genogroups (GI and GII) and as many as 20 subgroups. GII strains are responsible for most (73% of outbreaks from 1997–2000) NV cases in the US (Fankhauser *et al.* 2002) and 90% in Florida (L. Stark, personal communication). With an incubation period of only 24–48 h and as many as 30% of the infections being asymptomatic, this highly contagious virus often results in rapid disease transmission that affects large numbers of exposed individuals (Greene *et al.* 2003). For this reason, NV outbreaks are common in

closed environments such as daycare centers, nursing homes (Thornton *et al.* 2004) and cruise ships (Widdowson *et al.* 2004).

Acute gastroenteritis has also been identified as a significant cause of substantial morbidity for US military personnel during deployment (Oyofa *et al.* 1999). The most common disability amongst soldiers in Operation Desert Storm and Operation Desert Shield was gastroenteritis resulting from NV infection (McCarthy *et al.* 2000; Centers for Disease Control and Prevention 2002). Outbreaks of NV have also been commonly identified on US Naval vessels. From March to May 1996, 49 of 721 personnel aboard the USS Germantown reported to sick call with acute gastroenteritis, and 45% of the stool samples taken were positive for NV. From 1992 to 1997, four large outbreaks on Navy aircraft carriers were attributed to NV (McCarthy *et al.* 2000). NV also caused an acute outbreak of gastroenteritis amongst British soldiers deployed in

Afghanistan in May 2002 (Ahmad 2002; Centers for Disease Control and Prevention 2002). Although tainted food tends to be the primary mode of transmission, contact with contaminated surfaces and water play a significant role in the spread of this disease (Green 1997; Green *et al.* 1998; Barker *et al.* 2004).

NV is a significant worldwide public health concern and there is a need for rapid field detection and diagnosis such that patterns of transmission can be interrupted (Centers for Disease Control and Prevention 2002). This challenge is complicated by two main issues: (i) the inability to grow NV in culture and (ii) the high genetic diversity that exists among NVs. Nonetheless, as the cloning and sequencing of the first NV genome was completed (Jiang *et al.* 1990), several molecular methods have been published for detection of a myriad of NV genotypes (Greene *et al.* 2003; Jean *et al.* 2003; Hohne and Schreier 2004; Laverick *et al.* 2004; Moore *et al.* 2004).

The most common way to screen for NV is by reverse transcription-polymerase chain reaction (RT-PCR) followed by detection of the amplification product by gel electrophoresis or Southern hybridization (Greene *et al.* 2003). Building on this approach, a few reports of quantitative RT-PCR detection have been published (Jean *et al.* 2003; Kageyama *et al.* 2003; Hohne and Schreier 2004; Richards *et al.* 2004). These assays, however, require expensive equipment that may not be available to health-care providers and health departments. New to the US market, enzyme-linked immunosorbent assays have been used to detect NV, yet this approach has proven to be somewhat unreliable because of the highly diverse antigen profile from NV (Burton-MacLeod *et al.* 2004).

We have developed a real-time method to detect NV based on nucleic acid sequence-based amplification (NASBA) using a molecular beacon probe for detection. Although other NASBA assays have been developed for

detection of NV (Greene *et al.* 2003; Jean *et al.* 2004; Moore *et al.* 2004), these assays rely on end-point detection and therefore require more time for analysis. Furthermore, a real-time NASBA approach provides a semi-quantitative method to estimate viral load in as little as 20 min.

Materials and methods

In vitro transcript

To allow for an accurate estimate of copy number the assay could detect, the transcript was generated *in vitro* for the NV target sequence. Primers were designed according to sequences deposited in GenBank that were approximately 100 bases upstream and downstream of the SR46 and SR33 primer region described by Ando *et al.* (1995) (Table 1). Using the NVTF and NVTR primers, a 324 base region of the RNA-dependent RNA polymerase gene of NV GII was amplified from a NV isolate designated USF-NV by RT-PCR using the Access RT-PCR system (Promega Corporation, Madison, WI, USA) with an annealing temperature of 45°C. Reaction products were visualized on 2% agarose gels stained with ethidium bromide and subsequently TA TOPO cloned into pCRII (Invitrogen Corporation, Carlsbad, CA, USA) according to the protocol outlined by the manufacturer. Clones were sequenced using vector M13 priming sites to ensure the sequence integrity. A plasmid with the correct insert was linearized by digestion with *NotI*, the enzyme removed using the Wizard DNA clean-up kit (Promega Corporation), and run-off transcripts generated from an upstream SP6 promoter on the vector using the Riboprobe *in vitro* transcription kit (Promega Corporation). The transcripts were purified by the RNeasy spin protocol (Qiagen Corporation, Valencia, CA, USA) and quantified with a Ribogreen RNA quantification kit according to the manufacturer's instructions

Table 1 Primer and probe sequences for detection of noroviruses by conventional reverse transcription-polymerase chain reaction (RT-PCR), Taq-man RT-PCR and nucleic acid sequence-based amplification

| Primer | Sequence (5'–3') | Assay | Source |
|------------------------------|---|---------------------|-------------------------|
| NVTF | CCTTCACAGGCAAGTTGCCAGA | Generate transcript | This study |
| NVTR | CCATCATTAGATGGAGCGGCGTCA | Generate transcript | This study |
| SR46 | TGGAATCCATCGCCCACTGG | RT-PCR | Ando <i>et al.</i> 1995 |
| SR33 | TGTCACGATCTCATCACC | RT-PCR | Ando <i>et al.</i> 1995 |
| Nor3 | CAATGGAATTCCATCGCCCA | NASBA | This study |
| Nor4 | AATTCTAATACGACTCACTATAG GGAGAAGTTGTCACGATCTCATCATCA | NASBA | This study |
| Molecular beacon (NorBeacon) | [6-FAM]-CATCGGACATCATAAAGCTA ATTCCGATG-[DABCYL] | NASBA | This study |

6-FAM, 6-carboxy fluorescein.

(Molecular Probes Inc., Eugene, OR, USA). The transcript RNA was mixed 1 : 1 in an RNA storage buffer (8 mol l⁻¹ guanidinium isothiocyanate, 80 mmol l⁻¹ Tris-HCl (pH 8.5), 24 mmol l⁻¹ MgCl₂, 140 mmol l⁻¹ KCl) and frozen at -80°C until use.

Clinical samples

One hundred and thirty-two faecal samples from individuals that had been submitted for NV detection were obtained from the Florida Department of Health (FLDOH) Bureau of Laboratories (BOL) in Tampa. All samples were de-identified prior to being released from the BOL for this study, so not to be traceable to patient data.

Virus extraction from stool

To obtain a representative sample, frozen stools were thawed and a 10% suspension was prepared in phosphate-buffered saline (PBS). This suspension was mixed with an equal volume of 1,1,2-trichloro-1,2,2-trifluoroethane (Freon) and centrifuged at 1200 g for 10 min at 4°C. The aqueous phase was then removed for RNA extraction.

RNA extraction

Preliminary evaluation of each stool specimen was performed at the FLDOH by Trizol extraction, followed by RT-PCR. In brief, 250 µl of the 10% NV extract (aqueous phase) was mixed with 750 µl of Trizol (Invitrogen Corporation) and 200 µl chloroform, vortexed for 15 s and incubated at room temperature for 10 min. This mixture was then spun for 15 min at 12 000 × g at 4°C. The aqueous phase was precipitated with glycogen and isopropanol and the pellet washed with 75% EtOH. The final RNA pellet was resuspended in 10 µl of diethyl pyrocarbonate (DEPC)-treated H₂O and directly used in the RT-PCR reactions.

For all subsequent samples the RNA extraction was accomplished using the RNeasy Mini Kit (Qiagen Corporation). Briefly, 100 µl of the 10% NV extract (aqueous phase) was added to 350 µl RLT lysis buffer (Qiagen) and incubated at room temperature for 10 min. Subsequently, 250 µl of 100% EtOH was added and the sample was processed using the RNeasy spin column method as outlined by the manufacturer (Qiagen Corporation). The RNA was eluted from the column in 50 µl of DEPC-treated H₂O and used directly in the RT-PCR, Taqman or NASBA assays.

RT-PCR

RT-PCR was performed using the Access RT-PCR system (Promega Corporation). Each reaction was performed in

a final volume of 50 µl that contained 1X *Tfl*/AMV reaction buffer, 0.2 mmol l⁻¹ each dNTP, 1 mmol l⁻¹ MgSO₄, 5 U of *Tfl* DNA polymerase, 5 U of AMV RT, 1 µmol l⁻¹ of each primer (SR46 and SR33) and 5 µl of template RNA. The reactions were amplified using the following cycle parameters: an initial RT step was carried out at 45°C for 30 min followed by a denaturation step at 95°C for 10 min and 40 rounds of amplification with 1 min at 94°C, 1 min at 60°C and 1 min at 72°C. The resultant product (123 bp of the RNA-dependent RNA polymerase gene) was visualized on 2% agarose gels stained with ethidium bromide.

NASBA assay

NASBA was performed using the Nuclisens Basic Kit (bioMérieux, Durham, NC, USA) and an EasyQ incubator and detection system (bioMérieux). To reduce the cost of the assay, each sample was run in a 10 µl NASBA reaction (half the volume recommended by the bioMérieux protocol), consisting of 5 µl NASBA reagent/primer mix (80 mmol l⁻¹ KCl), 2.5 µl RNA template, and 2.5 µl enzyme mix (Nuclisens Basic Kit; bioMérieux). HPLC-purified primers and beacon were obtained from Qiagen, and were diluted to final concentrations of 400 nmol l⁻¹ for primers (Nor3 and Nor4) (Table 1) and 100 nmol l⁻¹ for each beacon per NASBA reaction. The NV beacon (NorBeacon) was labelled with 6-carboxy fluorescein (6-FAM) at its 5'-end and quencher Dabcyl at its 3'-end (Table 1).

Sequencing

Samples that were negative by NASBA or Taqman were subsequently rechecked by RT-PCR followed by detection on 2% agarose gels stained with ethidium bromide. If a visible product was present, it was TA TOPO cloned into the pCRII cloning vector according to the manufacturer's instructions and sequenced as described earlier using the M13 forward primer. Sequencing of clones was performed at the University of Florida sequencing facility using a Perkin Elmer/Applied Biosystems 373A or 377 sequencing system (Foster City, CA, USA). Some sequences were obtained by direct sequencing of RT-PCR products at the FLDOH (Tampa, FL, USA) using a Beckman-Coulter CE8000 Genetic Analysis System (Fullerton, CA, USA).

Swab samples

Freon extracted stool known to contain NV (10 µl) was spread into sterile petri dishes and allowed to dry. At times 1 h, 4 h and 4 days, the virus was resuspended by either polyester or cotton swabs saturated with 100 µl of

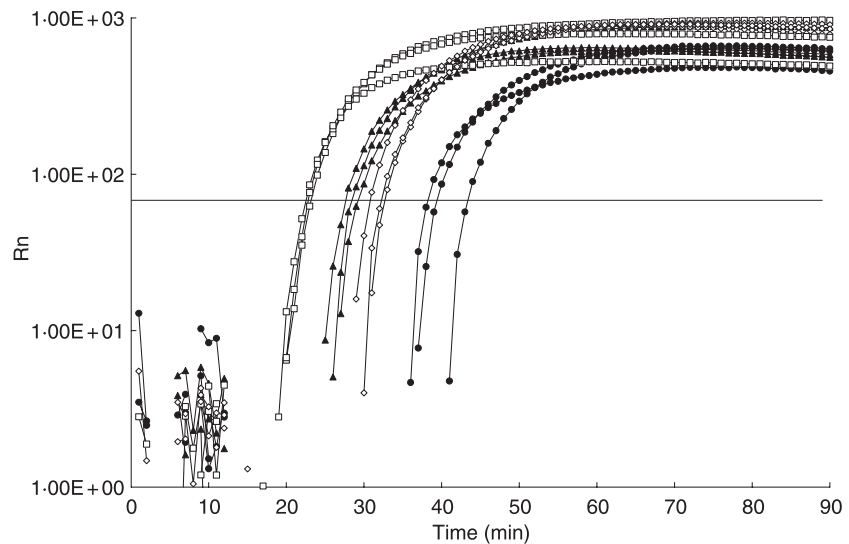


Figure 1 Amplification curves for norovirus transcript over four orders of magnitude. (■) 100 000 copies; (▲) 10 000 copies; (◇) 1000 copies and (●) 100 copies. Reactions were performed in triplicate.

DEPC-treated H₂O or RLT lysis buffer (Qiagen Corporation). Immediately following virus collection, the swab was placed into 350 μ l of RLT lysis buffer + 1% 2-mercaptoethanol for 10 min at room temperature and vortexed for 30 s. The RNA was then extracted using the RNeasy mini-spin kit according to the manufacturer's protocol (Qiagen Corporation). Control templates representing 100% recovery were obtained by a direct RNA extraction from 10 μ l of the Freon extracted stool containing NV and per cent recovery was calculated by comparison with a standard curve obtained from serial dilutions of this extraction. Standard curves were created using triplicate measurements at each dilution and plotting the time to positivity (TTP) measurement *vs* concentration.

Results

NASBA sensitivity

To allow for direct comparison of our results to the previously obtained results from the FLDOH, a molecular beacon-based NASBA assay was developed based on the RNA-dependent RNA polymerase gene for detection of GII isolates (Ando *et al.* 1995). To test the sensitivity of this assay, transcript generated from isolate USF-NV (FLDOH8, Fig. 4) was diluted and used as standards. Standard curves were generated using transcript serially diluted to between 100 and 100 000 copies of the target. NASBA amplification occurred in as little as 22 min for 10⁵ copies and all positive samples (>100 copies of target RNA) amplified (fluorescence two times background) within 50 min (Fig. 1), *vs* a minimum time requirement of 2.5 h with Taqman RT-PCR. There was a negative linear relationship ($R^2 = 0.94$) between the log number of

target RNA molecules and the TTP over at least four orders of magnitude (Fig. 2). The NASBA assay could routinely detect 1000 or more copies of the target RNA. Furthermore, 100 copies were detected in most runs (>80%) and as few as 10 copies occasionally amplified. These results were similar to the detection limits obtained using a real-time (Taqman) RT-PCR assay for NV (data not shown).

To further test the method, 129 stool samples previously determined to be NV positive by RT-PCR (by the FLDOH) and three negative stools from the FLDOH were analysed. Amplification was performed using the real-time NASBA assay described here. Of these faecal samples, 114 (88.4%) tested positive using NASBA.

Samples that tested incorrectly by NASBA were retested by conventional RT-PCR using the same conditions used

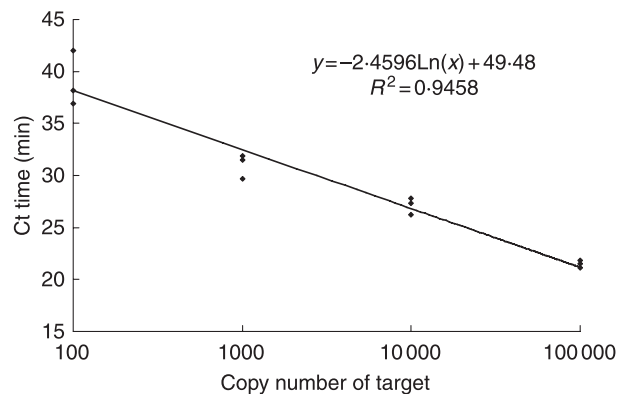


Figure 2 Standard curve of norovirus transcript (100–100 000 copies of target) using time to positivity measurements from the amplification curve data.

| Probe sequence | RT-PCR | TaqMan | NASBA | Number of samples |
|--|--------|--------|-------|-------------------|
| 1. G A C A T C - A T A C A G G C T A A T T C | + | - | + | 1 |
| 2. G A T A T G T A T A C A G G C C A A T T G | + | - | - | 1 |
| 3. A A C A T C - A T A C A G G C C A A T G G | + | + | - | 4 |
| 4. G A C A T C - A T G C A G G C A A A C T C | + | - | + | 2 |
| 5. C A T A T C C A T T C C G G C A A T T G G | + | - | - | 2 |
| 6. G A C A T C - A T C C A G G C A A A T T C | + | - | + | 1 |
| 7. A A C A T C - A T A C A G G C T A C A G T | + | - | - | 1 |
| 8. A A C A T C C A T A C A G G C T A A T T C | + | - | - | 1 |
| 9. G A T A A G C A T G C A G G C C A A T G T | + | - | - | 1 |

Figure 3 Sequence alignment of probe region from samples negative by Taqman reverse transcription-polymerase chain reaction, nucleic acid sequence-based amplification or both. Shaded areas indicate homologous nucleotides.

at the FLDOH. Although each sample produced a positive signal (123 bp product) by gel electrophoresis, some of the bands were faint. When possible these products were cloned and sequenced to assess the sequence diversity in the outbreak samples. Sequences were obtained from 11 of the 15 NASBA negative samples and four samples that were positive by NASBA but negative by a Taqman PCR assay designed to the same region. Alignments of the molecular beacon/Taqman probe region are shown in Fig. 3. A CLUSTAL analysis and neighbour joining tree was generated with sequences deposited in GenBank and sequences obtained in this study from both positive and negative NASBA reactions (Fig. 4). Although overall the samples tended to be split between two clades, the negative NASBA samples were evenly dispersed and no correlation with either clade was observed.

Recovery of NV from solid surfaces

To determine if detectable virus could be recovered from contaminated surfaces, Freon extracted stool known to contain virus was spread on a solid surface and allowed to dry. Virus recovery was evaluated at varying time points using polyester and cotton tipped swabs saturated with either DEPC-treated H₂O or RLT lysis buffer. Recovery of the virus was significantly higher when using the RLT paired with a polyester tipped swab *vs* the other treatments (*P* < 0.01) (Table 2). There was no significant difference between recovery after 1 h and 4 days for any of the treatments tested (Table 2).

Discussion

NV infections have long been recognized as a primary cause of viral gastroenteritis worldwide. To improve clinical diagnosis and provide better monitoring capabilities, a reliable detection system is needed. Unfortunately, the

high amount of genetic and antigenic diversity found in this virus has made this a significant challenge (Moore *et al.* 2004). Nonetheless, the ‘gold standard’ for NV testing has become RT-PCR followed by detection of the amplicon by gel electrophoresis or southern blotting (Vinje and Koopmans 1996). Unfortunately, these methods are time consuming and labour intensive. Furthermore, to assure environmental cleanup goals are met; fast, simple and portable detection methods are required. The goal of this study was to develop and evaluate a real-time NASBA based detection method for NV.

This work has established that a real-time molecular beacon based NASBA assay has the potential to be a sensitive rapid method for routine detection of NV. To our knowledge this is the first report of a real-time NASBA assay for NV. We were able to successfully amplify NV from contaminated stool samples with the same sensitivity and specificity as a real-time Taqman RT-PCR assay for the same region of the NV genome (data not shown). Assuming that each viral particle renders one copy of the target sequence, the NASBA assay was able to detect as few as 10 viral particles. Further, because this method uses a molecular beacon probe for detection, quantitative results for NV are possible. A negative linear relationship between the TTP and target concentration was obtained over four orders of magnitude (10⁵–10²) using diluted transcript as template RNA. However, slight sequence ambiguity in the primer and/or probe binding region can significantly alter the TTP result causing a lower predicted count for the viral load. Based on this knowledge and the high amount of genetic diversity that has been found in NV isolates, we conclude that this assay (as well as other molecular based NV assays to date) should be used as a qualitative or semi-quantitative assay for NV when the sequences of the samples are unknown.

Sequence analysis of some of the Florida outbreak specimens used in this study revealed that mismatches within

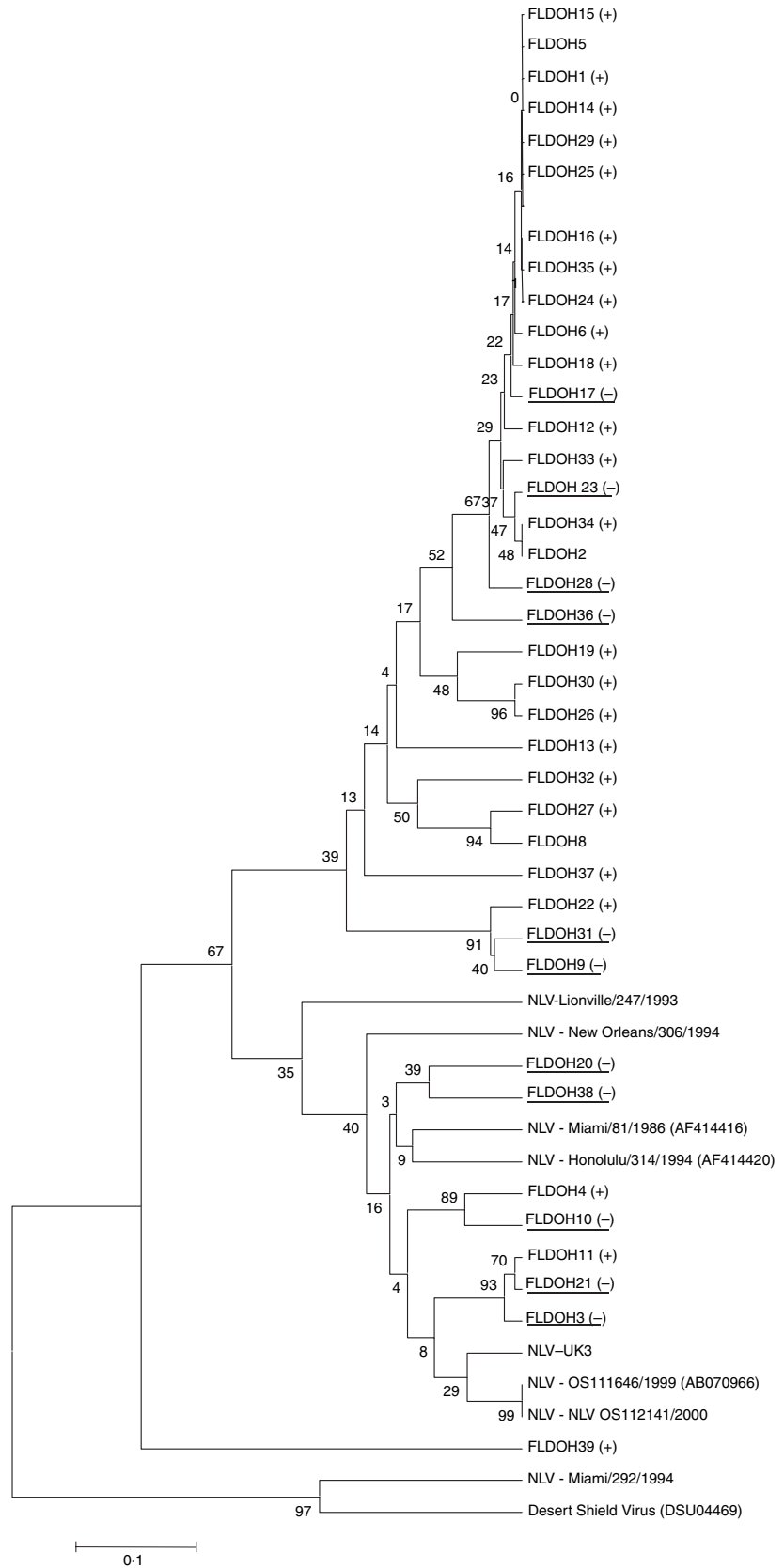


Figure 4 Neighbour joining tree based on sequence data of norovirus target region. Samples found to be positive by nucleic acid sequence-based amplification are denoted (+) and negative samples are underlined and contain (-).

Table 2 Per cent recovery of norovirus from a solid surface

| Swab type | Per cent recovery* | | |
|-----------------|--------------------|---------|--------|
| | 1 h | 4 h | 4 days |
| Cotton/water | 17 ± 9 | 22 ± 7 | 14 ± 5 |
| Cotton/RLT | 54 ± 2 | 52 ± 8 | 48 ± 8 |
| Polyester/water | 56 ± 8 | 58 ± 12 | 42 ± 7 |
| Polyester/RLT | 96 ± 5 | 101 ± 7 | 92 ± 9 |

*Per cent recovery calculated based on direct RNA extraction of virus. Each treatment performed in triplicate.

the molecular beacon probe binding region were present in many of the false negative samples. However, because these sequences tended to be diverse in regards to the mismatches, designing a broader reaching probe for this region of the NV genome may not be possible. One approach may be to mix several beacon sequences into a single reaction.

Because outbreaks of NV happen quickly and contaminated surfaces have been implicated as a significant mode of transmission (Green *et al.* 1998; Barker *et al.* 2004), we tested our ability to recover NV from solid surfaces. The best recovery of NV was achieved using polyester tipped swabs saturated with RLT lysis buffer. This proved to be a simple, yet effective, way to recover the virus even after extended periods of time (4 days) and therefore may be an effective way to sample for virus from suspect environmental surfaces. Further, this technology could be used with other NASBA assays for a variety of virus detection needs.

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