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Detection of Murine Norovirus-1 by Using TAT Peptide-Delivered Molecular Beacons▽†

Payal S. Ganguli,[1] Wilfred Chen,[2] and Marylynn V. Yates[1]*

Department of Environmental Sciences, University of California, Riverside, California 92521,[1] and Department of Chemical Engineering, University of Delaware, Newark, Delaware 19716[2]

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A TAT peptide-delivered molecular beacon was developed and utilized to enumerate murine norovirus 1, a human norovirus (NoV) surrogate, in RAW 264.7 cells. This allowed the detection of a single infective virus within 6 h, a 12-fold improvement in time required for viral detection and quantification compared to that required by the conventional plaque assay.

Human norovirus (NoV) causes significant negative health and economic impacts around the world (6, 7, 8). In the United States, norovirus has been implicated as the causative agent of 23 million cases of gastroenteritis and 50,000 hospitalizations annually (6, 10). Currently, no treatment or vaccine is available to prevent this disease. Attempts have been made to cultivate NoV in the laboratory, including a cell culture model employing three-dimensional (3D) human small intestinal epithelium cells (4, 13). This method is difficult to perform and not sufficiently robust for routine use (4, 13). Therefore, murine norovirus (MNV) has been increasingly used as a surrogate for human NoV, as MNV possesses similar biological and molecular properties (1, 18).

In this study, a molecular beacon (MB) targeting the MNV-1 virus was designed. Molecular beacons are increasingly used for real-time monitoring of virus replication in living cells. They have been used successfully in many *in vitro* hybridization assays due to their sensitivity and specificity and their ability to provide label-based and separation-free detection (5, 9, 15, 16, 19, 20). Molecular beacons are single-stranded, dually labeled antisense oligonucleotide-nucleotide probes having a stem-loop structure, with a fluorophore at one arm and a quencher at the other (5, 15) (Fig. 1). These highly target-specific probes produce fluorescence immediately upon binding, and they can distinguish single-nucleotide mismatches (9, 15, 20). Conjugation of the MB to the human immunodeficiency virus type 1 (HIV-1)-derived TAT peptide at the N terminus of the peptide using a thiol-maleimide linkage facilitates the traversing of biological membranes with nearly 100% efficiency. The TAT peptide-conjugated MB (TAT-MB) can be used to target complementary mRNA (2, 11, 14). In this study, a TAT-MB was developed to detect MNV-1 infection of RAW 264.7 cells.

Molecular beacon development. A 23-bp region of the 5′ untranslated region of MNV-1 virus was used as the target. The DNA backbone was modified with sulfur-substituted 2′-*O*-methyl oligoribonucleotides for improved nuclease resistance as described by Tsourkas et al. (14). Stem sequences were added, and mfold software (http://mfold.rna.albany.edu/?q=mfold/DNA-Folding-Form) and IDT SciTools were used to predict the thermodynamic properties and secondary structures of the MB. The MNV MB sequence is as follows: 5′-6-FAM-GCGACATGTCTGATTAGTGGGT TGGTTGTTGTC/thiol-dGC-DABCYL-3′ (where 6-FAM is 6-carboxyfluorescein) (stem sequence underlined). DABCYL [4-(4-dimethylaminophenylazo)benzoic acid] possessing a 2′-*O*-methylribonucleotide backbone with phosphorothioate internucleotide linkages was synthesized (TIB Molbiol, Inc.). A modified TAT peptide (H-Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg–N-$CHCH_2$–N-maleimide) (150 μM) was mixed with 100 μM thiolated MB in the dark for 2 h to form a stable thiol-maleimide linkage. The TAT-MB was stored at −20°C, and it was suspended in 100 mM Tris HCl (pH 8.0) buffer containing 1 mM $MgCl_2$ (final concentration, 100 μM) for subsequent studies.

Validation of TAT-MB properties. The TAT-MB's ability to detect MNV-1 infection of RAW 264.7 cells was tested by incubating 1 μM TAT-MB with the MB-complementary oligonucleotide MNV target (1:1) at 37°C in 5% CO_2 in an 8-well Lab-Tek chambered coverglass slide (Fisher Scientific, Inc.). Next, 1 μM MB without TAT was incubated with MNV-1-infected RAW 264.7 cells. Finally, a 1 μM nonspecific TAT-MB probe (5′-6-FAM-CGTGCGCGGAGCGGCTCG GAGGAGAAC/thiol-dG/CACG-DABCYL-3′), which was designed after performing NCBI-BLAST sequence searches, was incubated with MNV-1-infected cells to confirm the specificity of the TAT-MB probe.

Virus preparation and plaque assay. The MNV-1 stock and RAW 264.7 cells were prepared as described previously (1, 3, 12, 17). To determine the viral titer, RAW 264.7 cells (2×10^6 cells/plate) were seeded into six-well plates in complete Dulbecco modified Eagle medium (DMEM) (10% low-endotoxin fetal bovine serum, 1% L-glutamine, 1% penicillin-streptomycin, 1% HEPES) and incubated at 37°C in 5% CO_2 for 24 h until 90% confluence was achieved. Tenfold dilutions of MNV-1 samples were prepared in DMEM. Medium was aspirated from the confluent cells, and each well was inoculated with 0.1 ml virus solution. Three replicate assays per dilution were performed. Plates were incubated for 1 hour at room

* Corresponding author. Mailing address: Department of Environmental Sciences, University of California, Riverside, CA 92521. Phone: (951) 827-2358. Fax: (951) 827-3993. E-mail: marylynn.yates@ucr.edu.

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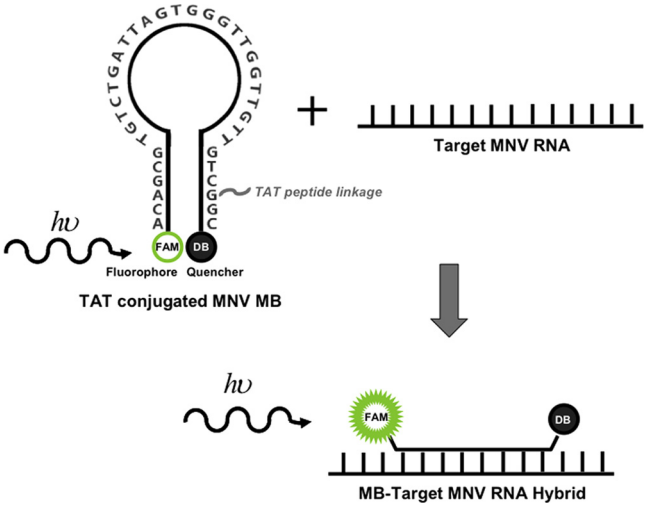

FIG. 1. Structure and mechanism of MNV-1 molecular beacon conjugated with TAT peptide. Molecular beacons are single-stranded oligonucleotide hairpin probes. In the presence of a complementary target RNA sequence, the beacon unfolds, hybridizes with the target, and exhibits fluorescence that is easily detectable.

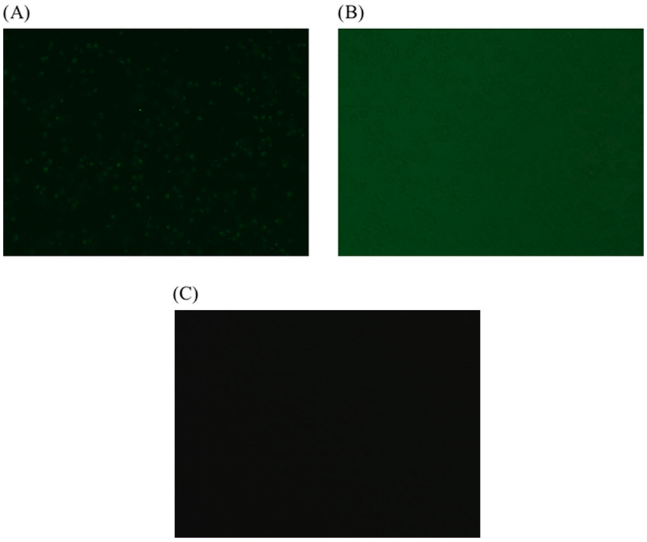

FIG. 2. Validation of the TAT-MB properties. (A) Presence of TAT peptide-conjugated MB probe and MNV-1 complementary target RNA; (B) absence of TAT, presence of nonconjugated MB probe in MNV-1-infected RAW 264.7 cells; (C) presence of nonspecific TAT-MB in MNV-1-infected RAW 264.7 cells. Images were acquired using a 10× objective.

temperature and gently rocked every 15 min. A 1:1 mixture of 1.5% SeaPlaque agarose and 2× minimum essential medium supplemented with 10% low-endotoxin fetal bovine serum, 2% L-glutamine, 2% penicillin-streptomycin, and 1% HEPES was overlaid onto the plates after incubation. The plates were reincubated at 37°C in a 5% CO_2 atmosphere for 48 h. To visualize plaques, cells were overlaid with 2 ml of a 1:1 mixture of 1.5% SeaKem agarose and complete 2× minimum essential medium (supplemented with 2% of a 3.3-g/liter stock solution of neutral red) per plate. After incubation for another 8 to 10 h at room temperature, the plaques were enumerated.

TAT-MB experiments. Eight-well chambered coverglass slides (monolayer of 5×10^6 RAW 264.7 cells/well) were incubated with 1 μM TAT-MB for 30 min at 37°C in a 5% CO_2 atmosphere in 1× Leibovitz L-15 medium (Invitrogen, Inc.). After incubation, the L-15 medium was aspirated, and the cells were washed twice with 1 M phosphate-buffered saline (PBS) (pH 7.4) and then infected with a 100-μl MNV-1 sample to obtain a multiplicity of infection (MOI) of 0.01 PFU/cell. RAW 264.7 cells incubated with 1 μM TAT-MB followed by infection with the MB-complementary oligonucleotide MNV target was used as a positive control. For the negative control, RAW 264.7 cells were incubated with a 1 μM nonspecific TAT-MB probe, followed by MNV-1 infection as described above. MNV-1 plaque assays were done for comparison purposes in triplicate.

To assess the application of this method to environmental water samples, 10^5 PFU MNV-1 was seeded into a 50-ml groundwater sample obtained from a Southern California water utility. This was diluted in 1× L-15 medium to obtain an MOI of 0.01 PFU/cell when added to confluent monolayers of RAW 264.7 cells. Three replicates per dilution were used for microscopy and plaque assays.

Cell imaging. For real-time experiments, when positive fluorescent signals were observed, the slide was placed on the microscope stage and several fluorescent images were taken until 12 h postinfection (hpi). Live-cell imaging was performed with an Olympus 1X71 inverted fluorescence microscope equipped with a 19-V, HBO 100-W/AC mercury lamp and a multiband dichromatic mirror. 6-FAM-labeled molecular beacon fluorescent hybrids were detected using a D480/30 nm exciter–D535/40 nm emitter filter set. Fluorescent cells were imaged and counted using Image-Pro Plus software (Media Cybernetics, Inc., Bethesda, MD).

The results of the experiments to assess the proper functioning of the TAT-MB are shown in Fig. 2. After incubation with the MB-complementary oligonucleotide MNV target, fluorescence was detected in 100% of the cells after 30 min (Fig. 2A). The fluorescence intensity remained constant for up to 12 h, validating the TAT-MB's target specificity and its resistance to intracellular nuclease activity. In the absence of TAT, no internalization of MB was observed; therefore, fluorescence was detected only in the medium (Fig. 2B). Introduction of the nonspecific TAT-MB into infected RAW cells produced no detectable fluorescence, validating the specificity of the TAT-MB (Fig. 2C).

Visualization of fluorescent cells increased with increasing time of infection with MNV-1 in RAW 264.7 cells in 1× Leibovitz L-15 medium (Fig. 3). After 12 hpi, 100% fluorescence was observed, the result of the onset of secondary infection due to the release of mature virions during cell lysis.

A linear correlation ($r^2 = 0.9821$) between the number of fluorescent cells and the number of PFU was observed at different concentrations of MNV-1 in RAW 264.7 cells at 6 hpi for three replicate experiments (Fig. 4). However, the plaque assay required 72 h to detect 1 PFU of MNV-1, compared to the 6-h detection time for the TAT-MB assay.

In groundwater containing 0.01 MOI of MNV-1, 100% fluorescence was obtained after 12 h of infection (Fig. 5). One

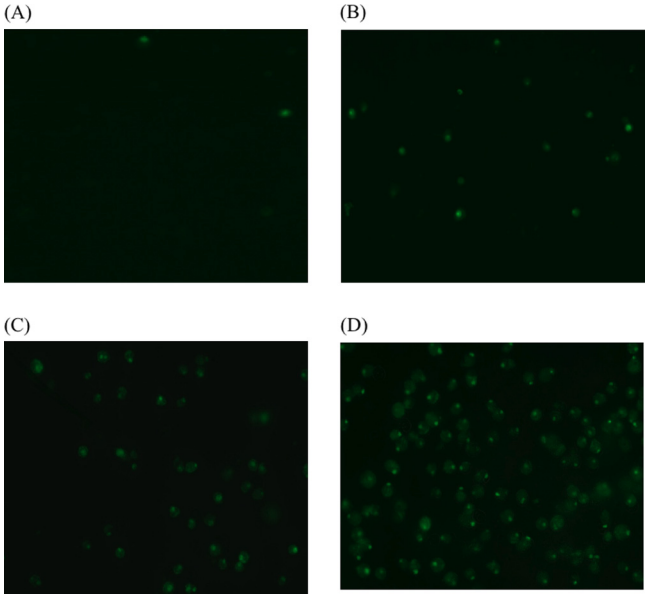

FIG. 3. Progression of MNV-1 infection in 1× Leibovitz L-15 medium. RAW 264.7 cells were infected with MNV-1 at an MOI of 0.01 PFU/cell. (A) 6 hpi; (B) 8 hpi; (C) 10 hpi; (D) 12 hpi. Images were acquired using a 20× objective.

PFU could be detected within 6 h, but a longer incubation time was chosen to confirm low virus numbers and to assess the TAT-MB probe's stability.

This study demonstrated the use of TAT-MBs as a rapid tool for detecting and quantifying infective MNV-1. Due to the rapid intracellular delivery of TAT-MB, the hybridization of viral RNA occurs shortly after virus uncoating, enabling virus detection early in its replication cycle (19, 20). The progress of MNV-1 infection was followed in real time by infecting RAW 264.7 cultures with MNV-1 (MOI = 0.01; see the movie in the supplemental material), and infected cells were detected within 6 hpi, a 12-fold time improvement over the plaque assay, with a similar quantitative ability. This approach may be applied to the real-time detection of infectious human norovirus once a suitable cell line is identified, as well as other difficult-to-culture viruses.

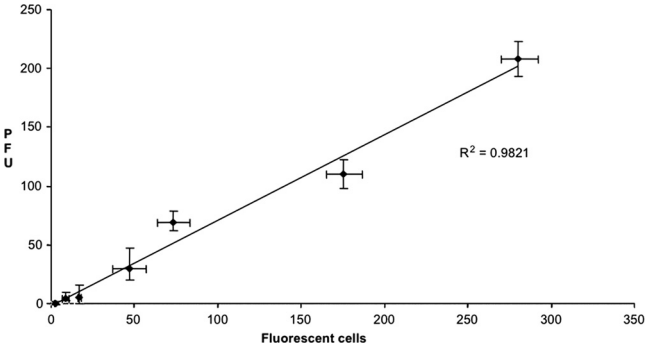

FIG. 4. Comparison of number of PFU versus number of fluorescent cells. Correlation of results obtained by conventional plaque assay (PFU) versus fluorescent cells obtained by molecular beacon assay at different concentrations of MNV-1 in RAW 264.7 cells at 6 h postinfection.

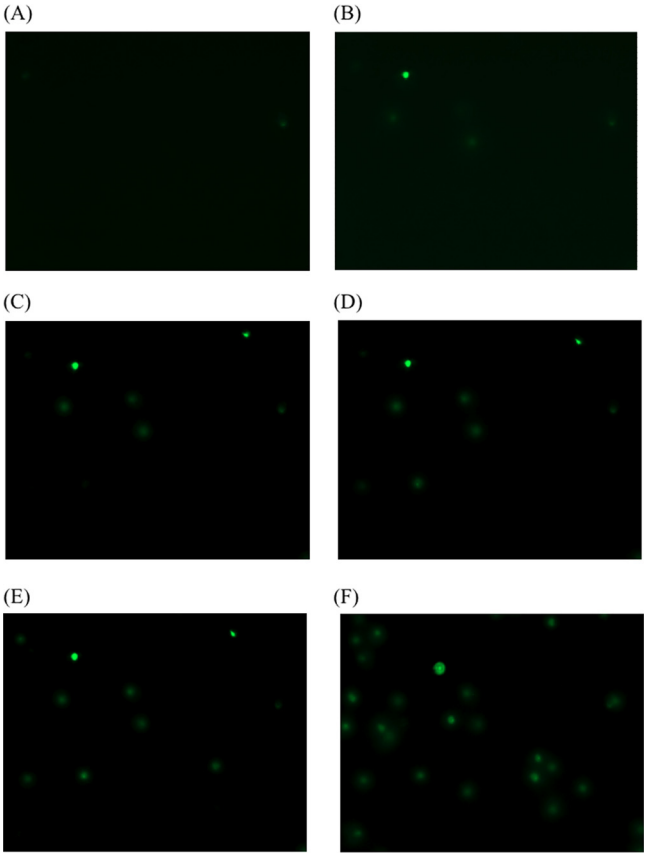

FIG. 5. Progression of MNV-1 infection in RAW 264.7 cells at an MOI of 0.01 inoculated into a groundwater sample. (A) 6 hpi; (B) 7 hpi; (C) 8 hpi; (D) 9 hpi; (E) 10 hpi; (F) 12 hpi. Images were acquired using a 20× objective.

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