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Histo-Blood Group Antigen Assay for Detecting Noroviruses in Water[▽]

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We evaluated a novel, magnetic-bead-based histo-blood group antigen assay for the recovery of low numbers of norovirus particles. Using this assay, with Norwalk virus seeded in environmental waters as a model, we were able to recover 30 to 300 genomic copies of the virus.

In recent years, human noroviruses (NoVs) have been recognized as the leading cause of outbreaks of acute gastroenteritis worldwide. Large NoV outbreaks have been reported in association with water used for drinking, recreation, shellfish harvesting, irrigation, and washing produce (1, 2, 3, 5, 7, 10, 13, 16, 20). NoVs recognize human histo-blood group antigens (HBGAs) as receptors (4, 12, 14). Since NoVs cannot be cultured in vitro, the virus is detected by reverse transcription-PCR (RT-PCR) assays, which are susceptible to inhibitors often found in environmental waters. Here, we report a novel method for the rapid recovery of low numbers of NoVs by the use of a magnetic-bead-based HBGA assay.

HBGA binding assay optimization. Using a previously described HBGA binding assay (9) we evaluated three blocking buffers (5% [wt/vol] Blotto, 5% [vol/vol] fetal bovine serum, and SuperBlock [Pierce Biotechnology, Rockford, IL]) for their ability to block nonspecific binding to uncoated magnetic beads without interfering with specific binding of virus to HBGA or interfering with the reproducibility of results. Based on these criteria, SuperBlock blocking buffer was chosen for subsequent experiments. In brief, 25 μl of washed MyOne streptavidin-coated magnetic beads (Dynal Biotech, Olso, Norway) was incubated for 90 min at room temperature with 50 μl of synthetic biotinylated H type 1 HBGA (1 mg/ml) (GlycoTech, Rockville, MD), followed by overnight incubation with blocking buffer. RNA copy numbers of the Norwalk virus (NV) stool suspensions were determined by comparison to a standard curve, using NV strain 8FIIb RNA transcripts. Dilutions of 10% stool suspensions containing 3,000, 300, 30, or 3 NV (8FIIb) copy numbers were added to 1 ml of blocking buffer, environmental water concentrate, or phosphate-buffered saline prior to incubation (4 h at room temperature) on an end-over-end rotator (Dynal Biotech). After eight washes, the beads were suspended in 50 μl of phosphate-buffered saline and subjected to heat (5 min at 99°C) to release the viral RNA. For the HBGA assay, 2.5 μl or 1 μl of heat-released NV RNA was analyzed by use of a conventional RT-PCR (Qiagen OneStep RT-PCR kit; Qiagen, Valencia, CA) (6) or a TaqMan real-time RT-PCR (QuantiTect probe RT-PCR kit; Qiagen) on an Applied Biosystems 7500 real-time PCR system platform (Foster City, CA) (22). The detection limit for the real-time assay was 10 RNA copy numbers.

The method is sensitive and specific for detecting NoV. A median of 300 copy numbers ($n = 10$) per milliliter of blocking buffer was detected by the HBGA assay (Table 1). In the presence of other enteric viruses (rotavirus group A serotype 1 [strain WA] or human astrovirus type 1 [Oxford strain]), 300 NV copy numbers were recovered by the assay ($n = 2$).

The method is sensitive in the context of environmental waters. Surface water ($n = 4$) and influent ($n = 2$) and effluent ($n = 4$) wastewater samples were collected from drinking-water and wastewater facilities in Columbus and Atlanta, GA, respectively. Surface water samples were concentrated by precipitation with 8% polyethylene glycol (PEG) 8000 and 0.3 M NaCl (23). After centrifugation (7,280 × *g*, 30 min), the PEG pellet was extracted with 50% (vol/vol) chloroform. Supernatants (5 to 20 ml) were stored at −70°C until use. The wastewater samples (850 ml) were processed similarly; only an additional centrifugation step (7,280 × *g*, 15 min at 4°C) and chloroform extraction of the pellet (50% [vol/vol]) were performed prior to PEG precipitation of the combined aqueous phases. Water concentrates were seeded with 3, 30, 300, or 3,000 copy numbers of NV and analyzed, using the HBGA binding assay. The detection limit for each water type tested was between 30 and 3,000 (median, 300) (Table 1).

The method detects viruses with an intact capsid. To determine if the NV particles were intact and potentially infectious, we bound 30,000 or 6,000 NoV copy numbers to H type 1 HGBA-coated beads before treatment with 10 ng of RNase A (Invitrogen, Carlsbad, CA) in Tris-EDTA buffer and incubation for 30 min at 37°C. To neutralize any remaining RNase activity, RNase inhibitor (160 U/50 μl sample) (Invitrogen) was added, and the sample was incubated for 15 min at 37°C. Before and after RNase treatment, GII.4 RNA (1×10^5 copy numbers) was added to each reaction mixture to check the efficacy of the RNase A or the RNase inhibitor. The mean cycle threshold values for the treated (35.8; range, 34.5 to 37.0) and untreated (35.2; range, 34.5 to 35.9) samples did not differ significantly ($P \geq 0.2$; Student's *t* test) at either input level. As expected, RNase treatment of the GII.4 control RNA resulted in complete loss of signal, when analyzed by TaqMan real-time RT-PCR (22).

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TABLE 1. Detection limits of the HBGA binding assay for Norwalk virus in the presence of an environmental water matrix or SuperBlock blocking buffer

Type of environmental water or control	No. of samples detected/total no. of samples at each indicated input level (RNA copy no.)			
	3,000	300	30	3
SuperBlock control	10/10	9/10	4/10	1/10
Surface water	4/4	4/4	4/4	0/4
Effluent wastewater	4/4	4/4	0/4	0/4
Influent wastewater	2/2	1/2	0/2	0/4

In summary, we report a novel HBGA magnetic bead separation method for human NoVs. The method is sensitive and specific for detecting NoVs with an intact HBGA receptor binding site. Furthermore, since we demonstrated only a 1-log decrease in method sensitivity upon application to sewage samples, this assay can be used to remove RT-PCR inhibitory compounds present in environmental waters.

Several research groups have developed sensitive methods for concentrating NoVs by use of immunomagnetic separation or gastric mucins from pigs (8, 17, 18, 19, 21). Our method can detect low numbers of NoVs, but it also detects NoV bound to its specific HBGA receptor, which may be a surrogate for detecting infectious virus. Previous studies indicate that many, but not all, NoVs bind to HBGAs (9, 11, 15), and the requirement of a secondary receptor during infection is not clear. Elucidating the role of HBGA and NoV infectivity will be needed to further validate the value of this assay as a surrogate for detecting infectious virus. In conclusion, we report an assay that may serve as a rapid detection method for potentially infectious NoVs in complex matrices, such as environmental waters.

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