An ultrasensitive and fully automated bioluminescent enzyme immunoassay (BLEIA) was developed for the detection of norovirus (NV) capsid antigen. In the evaluation tests with recombinant virus-like particles, the BLEIA demonstrated broad reactivity against several NV genotypes (genotypes 1, 3, 4, 7, 8, and 12 in genogroup I [GI] and genotypes 1, 2, 3, 4, 5, 6, 12, and 13 in GII), a wide dose-response range from 0.25 pg/ml to 10,000 pg/ml, and good reproducibility with low coefficients of variation (CVs) (within-run CVs of <2.8%, between-day CVs of <3.7%). In the evaluation tests with NV-positive fecal samples, a good correlation ($y = 0.66x - 3.21, r = 0.84$) between the BLEIA and real-time quantitative reverse transcription-PCR was obtained. Furthermore, in the dilution test with NV specimens, the analytical sensitivity of NV was estimated to be $10^5$ to $10^6$ copies/g of fecal sample, indicating that the analytical sensitivity of the BLEIA is comparable to that of commercially available molecular methods. All assay steps are fully automated, the turnaround time is 46 min, and the throughput of the assay is 120 tests/h. These results indicate that the BLEIA is potentially useful for the rapid diagnosis of NV in epidemic and sporadic gastroenteritis.

Noroviruses (NVs), which belong to the family *Caliciviridae*, are the leading cause of nonbacterial acute gastroenteritis in humans worldwide (5, 8, 17). NV is extremely contagious and spreads easily by multiple modes of transmission. NV outbreaks often occur in semiclosed communities that promote person-to-person transmission through the fecal-oral route, including schools, hospitals, long-term-care facilities, and cruise ships (6, 13). Because the NV infectious dose is reportedly less than 100 viral particles (30), sensitive and rapid diagnosis is crucial to prevent an NV outbreak due to person-to-person transmission.

NV is highly diverse genetically and has been divided into five genogroups, and most human strains belong to two of these: genogroup I (GI) and genogroup II (GII). Furthermore, these genogroups have been classified into at least 26 genotypes (12, 20, 24, 31). Although these levels of genetic diversity make it difficult to design broadly reactive primers, conventional reverse transcription-PCR (RT-PCR) and real-time RT-PCR have been developed and are widely used for the detection of NV (11, 16). They are reliable techniques for highly sensitive detection of NV from various specimens, including stool, vomitus, food, and water. However, they have some disadvantages in that they are time-consuming and not easy to perform.

Compared with these molecular methods, conventional immunological methods, such as enzyme linked immunosorbent assay (ELISA) and the immunochromatographic (IC) test, are thought to be simpler methods for the diagnosis of NV infection. To date, several ELISA and IC kits have been developed and widely used for the rapid diagnosis of norovirus gastroenteritis. However, they are reported to be inadequate to replace molecular methods due to their moderate sensitivity (3).

Recently, a bioluminescent enzyme immunoassay (BLEIA) was developed by using firefly luciferase as a label (18, 23, 27). Firefly luciferase is suitable for highly sensitive detection due to its high quantum yield, so that the BLEIA provides highly sensitive analyses of the target molecules. Judging from the performance of the BLEIA, it should be possible to construct an ultrasensitive enzyme immunoassay for NV that has nearly equivalent sensitivity to molecular methods. Therefore, we attempted to construct a fully automated BLEIA for NV by using firefly luciferase and antibodies reactive to a wide variety of NV genotypes.

**MATERIALS AND METHODS**

**Specimen collection.** After informed consent was obtained, a total of 171 fecal samples were collected from pediatric patients with sporadic gastroenteritis in Kanagawa and Tochigi Prefectures in Japan between October 2008 and February 2010. Similarly, after informed consent was obtained, a total of 489 fecal samples were collected from adult healthy volunteers from our company in Tochigi Prefecture in Japan between December 2006 and November 2010. All of the fecal samples were stored at −80°C until tested.

**Real-time RT-PCR for detection of NV.** A total of 171 fecal samples collected from pediatric patients were assayed by real-time RT-PCR. Ten percent (wt/vol) fecal suspensions were prepared with phosphate-buffered saline and centrifuged at 13,000 $\times g$ for 20 min. Viral RNA was extracted from the fecal supernatant using a QIAamp viral RNA kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions, and cDNA was synthesized by Superscript III first-strand synthesis (Invitrogen Carlsbad, CA). The real-time RT-PCR was performed according to the report described previously (11). After purification of the real-time RT-PCR-positive products with a QIAquick PCR purification kit (Qiagen, Hilden, Germany), the sequencing of 260 bp was performed by Bio Matrix Research (Chiba, Japan). The sequences were compared with those of the reference strains of NV obtained from GenBank and classified into 31 genotypes as described by Kageyama et al. (12). We successfully quantified the viral loads of 61 out of 171 fecal samples and correctly determined their genotypes. All 61 samples were assayed with the BLEIA.
Preparation of NV VLPs. Fourteen genotypes of NV virus-like particles (VLPs) (genotypes 1, 3, 4, 7, 8, and 12 in GI and genotypes 1, 2, 3, 4, 5, 6, 12 and 13 in GII) were prepared from NV isolated from stool samples. These stool samples were kindly provided by Shinji Fukuda (Hiroshima Prefectural Technology Research Institute). The production of recombinant bacmids was performed using the Bac-to-Bac baculovirus expression system with Gateway Technology (Invitrogen Carlsbad, CA), and the transfection of bacmids into insect cells of the system with Gateway Technology (Invitrogen Carlsbad, CA), and the transfection of bacmids into insect cells of the Spodoptera frugiperda line SF21 was performed as described previously (2,10). The VLPs from the insect cells were purified by ultracentrifugation through a sucrose density gradient. Protein concentrations of purified VLPs were determined with a bicinchoninic acid (BCA) assay reagent kit and bovine serum albumin (BSA) as a standard (Thermo Fisher Scientific, Waltham, MA).

Production of monoclonal antibodies. GL4 and GIIL.6 VLPs were employed as immunogens. The ddY mice were immunized with 50 μg of each VLP 5 times intraperitoneally. The first four immunizations were done with an emulsion of complete Freund’s adjuvant. The final immunization was done with an emulsion of incomplete Freund’s adjuvant. Spleen cells from the immunized mice were fused with NS-1 myeloma cells. The resultant hybridoma cells were selected in hypoxanthine-aminopterin-thymidine (HAT) selection medium. Anti-NV capsid protein antibody-producing hybridomas were selected by the VLP-immobilized ELISA and cloned by limiting dilution. VLP-immobilized ELISA was performed as follows. Microwells were coated with 0.15 μg of VLPs per well in 50 μL of phosphate-buffered saline (PBS) for 1 h at room temperature. Each well was washed with PBS–0.05% Tween 20 (PBS-T) and postcoated with 25% Block Ace (Dainippon Sumitomo Pharma, Osaka, Japan) overnight at 4°C. One hundred microliters of the hybridoma supernatant was added to each well, and the mixture was incubated for 1 h at 37°C. After being washed with PBS-T, 50 μL of anti-mouse IgG-horseradish peroxidase (HRP) conjugate, diluted 1:2,500 with PBS-T, was added and the mixture was incubated for 1 h at 37°C. After being washed, 100 μL of the substrate solution containing 1 mM H₂O₂ and 0.25 M 3,3',5,5'-tetramethylbenzine (Dojindo Laboratories, Kumamoto, Japan) was added to each well, and the mixture was incubated for 20 min at room temperature. The reaction was stopped by adding 100 μL of 3 N sulfuric acid to each well, and the optical density at 492 nm (with that at 620 nm as the reference) was measured.

Immunization of monoclonal antibodies on magnetic particles. Murine anti-norovirus capsid antigen monoclonal antibodies 107-5 and v6-29 were immobilized onto magnetic particles through the carbodiimide method (19). The carboxyl magnetic particles (JSR, Tokyo, Japan) were washed with distilled water. After being washed, the particles were activated with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (Dojindo Laboratories, Kumamoto, Japan) at 25°C for 30 min with agitation. After being washed, the particles were mixed with IgG 107-5 and v6-29 at 4°C for 16 h. After immobilization, the particles were washed and postcoated with distilled water containing 0.001% Triton X-100 and 0.5% BSA at 25°C for 3 h. The Fab′ fragment was purified by gel filtration on Superdex 200HR equilibrated with 50 mM phosphate buffer (PB) (pH 6.0) containing 500 mM NaCl and 0.1% BSA at a particle concentration of 0.13% (wt/vol), and stored at 4°C.

Preparation of luciferase-labeled conjugate. Murine anti-norovirus capsid antigen IgG monoclonal antibodies m9-110 and 212-60-5 were digested by pepsin (Sigma, St. Louis, MO) in 0.1 M acetic buffer at pH 4.0, and the Fab′ fragment was purified by gel filtration on Superdex 200HR (GE Healthcare, Buckinghamshire, United Kingdom) equilibrated with 100 mM PB at pH 7.2. The Fab′ fragment was prepared by reducing the Fab′ fragment, and conjugated to streptavidin (MP Biomedicals, Irvine, CA) by a method described previously (18). The Fab′-streptavidin conjugate was purified by gel filtration on Superdex 200HR equilibrated with 50 mM PB containing 5 mM EDTA at pH 7.0. After purification, the Fab′-streptavidin conjugate was mixed with biotinylated firefly luciferase (Kikkoman Biochemifa, Tokyo, Japan) at a molar ratio of 1:1. The mixture was incubated at room temperature for 1 h to form an antibody-luciferase complex. The resulting luciferase-labeled conjugate was diluted in 100 mM PB (pH 6.7) containing to 300 mM NaCl and 0.5% BSA at a final luciferase concentration of 6.5 mM.

Bioluminescent enzyme immunoassay. The bioluminescent enzyme immunoassay protocol is illustrated in Fig. 1. All assay steps were performed with the automated bioluminescent enzyme immunoassay analyzer BLEIA-1200 (Eiken Chemical, Tokyo, Japan). Two hundred microliters of sample, 190 μl of reaction buffer 1 (260 mM Tris-HCl, 0.1% sodium N-dodecanoyl salcosinate [pH 8.8]), 100 μl of reaction buffer 2 (260 mM Tris-HCl, 3.5% BSA [pH 8.8]), and 20 μl of the suspension of antibody-immobilized magnetic particles (MP) were mixed. The mixture was incubated at 37°C for 15 min for the first reaction. After the first reaction, MP was washed five times with 0.05% Tween 20 in PBS. After being washed, 20 μl of luciferase-labeled conjugate and 100 μl of diluent (100 mM phosphate, 0.2% BSA [pH 6.8]) were added to the MP. The mixture was incubated at 37°C for 15 min for the second reaction. After
the second reaction, MP was washed in a similar manner as described above. Thereafter, 100 μl of substrate solution (40 mM N-(2-acetamido)-iminodiacet acid [ADA], 16 mM MgSO4, 2 mM ATP, 940 μM luciferin, 0.26 mM EDTA, 20 mM potassium pyrophosphate [pH 7.4]) was added to MP. Luminescent intensity was measured by integrating the emission for 5 s (delay time of 0.5 s) using a photomultiplier tube-based detector equipped with BLEIA-1200. The turnaround time is 46 min, and the throughput of the assay is 120 tests/hour.

The dose-response curve of the BLEIA was determined using serially diluted GII.4 VLP solutions ranging from 0.25 pg/ml to 10,000 pg/ml. The lowest dose response was defined as the antigen concentration at which the mean luminescent intensity = 2.6 standard deviations (SD) did not overlap the mean luminescent intensity + 2.6 SD for the zero calibrator.

The reproducibility of the BLEIA was evaluated using three GII.4 VLP panels with different concentrations of protein (A, B, and C). For within-run reproducibility, these panels were measured by the BLEIA in multiplexes (n = 10). For between-day reproducibility, these panels were measured by the BLEIA on 5 successive days. The concentration of VLP in each panel was determined using a previously generated calibration curve.

The reproducibility of the BLEIA to various genotypes was evaluated using 14 genotypes of VLPs (genotypes 1, 3, 4, 7, 8, and 12 in G1 and genotypes 1, 2, 3, 4, 5, 6, 12, and 13 in GII). Each VLP solution prepared to a concentration of 1.0 ng/ml was measured by the BLEIA in triplicate.

To evaluate the interference from fecal elements in the BLEIA, bovine serum albumin (100 μg/ml), glucose (100 μg/ml), soybean oil (4%), ascorbic acid (100 μg/ml), and enteric bacteria (1.0 × 108 CFU/ml), including Salmonella enterica serovar Enteritidis, Bacillus cereus, Campylobacter coli, Campylobacter jejuni, Citrobacter freundii, Chlostridium perfringens, Enterococcus faecalis, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa, Vibrio parahaemolyticus, Candida albicans, Escherichia coli, and Bacteroides fragilis, were investigated. Each of the GII.4 VLP solutions described above containing each of the interference substances at a concentration of 25 pg/ml was assayed by the BLEIA.

**Determination of cutoff value.** Two percent (wt/vol) fecal suspensions were prepared with sample buffer (10 mM MES [morpholineethanesulfonic acid], 1.5% BSA, 10 mM EDTA, pH 6.0) using 489 fecal samples collected from healthy volunteers. The supernatants were centrifuged at 22,000 × g for 10 min and measured by the BLEIA. The cutoff value was determined as 3.5 × the mean luminescent intensity of 489 fecal samples collected from healthy volunteers.

**Measurement of NV specimens using the BLEIA.** Two percent (wt/vol) fecal suspensions were prepared with sample buffer using 61 fecal samples confirmed to be NV positive by real-time RT-PCR. The supernatants were collected by centrifugation at 22,000 × g for 10 min and measured by the BLEIA. Individual index values were calculated by the formula: luminescent intensity of sample tested/cutoff value in each assay. Samples with an index value greater than 1.0 were regarded as positive, and samples with an index value lower than 1.0 were regarded as negative.

To determine the analytical sensitivity of the BLEIA, the dilution test was performed. One percent NV-positive fecal supernatants quantified by real-time RT-PCR were 10-fold serially diluted with sample buffer. The diluted supernatants were assayed with the BLEIA. The number of RNA copies by real-time RT-PCR in the highest dilution positive was used to calculate the virus concentration per gram of fecal sample.

**RESULTS**

**Dose-response curve of the BLEIA.** The dose-response curve was determined using the serially diluted GII.4 VLP standard (Fig. 2). The upper limit of the dose response was approximately 10,000 pg/ml. The lowest dose response was determined to be 0.25 pg/ml. As shown in Fig. 2, a good linearity was obtained from 1 pg/ml to 10,000 pg/ml. Although the linearity below 1 pg/ml is not good, the BLEIA demonstrated a wide dose-response range from 0.25 pg/ml to 10,000 pg/ml.

**Reproducibility of the BLEIA.** Reproducibility was evaluated using three GII.4 VLP panels (A, B, and C). The results are shown in Table 1. Within-run CV ranged from 1.4 to 2.8%. Between-day CV ranged from 1.9 to 3.7%. The BLEIA demonstrated good reproducibility, with low CV of less than 3.7% in both within-run and between-day reproducibility tests.

**Broad reactivity of the BLEIA to various NV genotypes of VLPs.** The BLEIA reacted with all 14 genotypes of VLPs prepared for this study (Fig. 3). Compared with GII.4, GII.1 and GII.3 showed higher reactivity. On the other hand, the other genotypes showed lower reactivity. The highest was GII.1, which showed 2-fold higher reactivity than GII.4. The lowest was GII.7, which showed about 10-fold lower reactivity than GII.4.

**Interference from fecal elements in the BLEIA.** The influence of fecal elements on the assay was evaluated using bovine serum albumin (100 μg/ml), glucose (100 μg/ml), soybean oil (4%), ascorbic acid (100 μg/ml), and enteric bacteria (1.0 × 108 CFU/ml). In the BLEIA, we observed no interference from these elements (data not shown).

**Determination of cutoff value.** In order to determine the cutoff value of the BLEIA, the distribution of fecal samples from healthy volunteers (n = 489) was measured (Fig. 4). The mean luminescent intensity of fecal samples was 1,918 relative light units (RLU), and the SD was 403 RLU. The cutoff value was de-

| TABLE 1 Reproducibility study values* | Sample Mean ± SD VLP concn (pg/ml) CV (%) |
|--------------------------------------|------------------------------------------|
| Within-run reproducibility (n = 10)  | A  9.8 ± 0.3  2.8                        |
|                                      | B  94.9 ± 1.4  1.5                       |
|                                      | C  957.4 ± 13.0  1.4                     |
| Between-day reproducibility (n = 5)  | A  9.7 ± 0.2  1.9                        |
|                                      | B  91.6 ± 3.4  3.7                       |
|                                      | C  925.7 ± 23.2  2.5                      |

* Reproducibility was assessed using different concentrations of VLP panels for within-run reproducibility and between-day reproducibility.
to the mean luminescent intensity, which is equivalent to the mean luminescent intensity + 12 SD (6,760 RLU). It was also confirmed that the cutoff value was equivalent to the luminescent intensity of 1 pg/ml GI.4 VLP standard.

Correlation with real-time RT-PCR. The correlation between the BLEIA and real-time RT-PCR was evaluated using the NV specimens from pediatric patients with sporadic gastroenteritis (n = 61). Of the 61 NV specimens, 54 were positive for GI (GI.4, 47.5% [29/61]; GI.2, 13.1% [8/61]; GI.3, 11.5% [7/61]; GI.6, 11.5% [7/61]; GI.13, 3.3% [2/61]; GI.1, 1.6% [1/61]) and the others were positive for GI (GI.4, 6.6% [4/61]; GI.2, 1.6% [1/61]; GI.7, 1.6% [1/61]; GI.8, 1.6% [1/61]). The virus concentration per gram of fecal sample was measured by real-time RT-PCR, and the viral load was compared with the index value of the BLEIA. As a result, a good correlation between the BLEIA and real-time RT-PCR was obtained (y = 0.66x − 3.21, r = 0.84, P < 0.001), as shown in Fig. 5. When evaluated in the respective genogroup, GI (n = 54) showed a good correlation between the BLEIA and real-time RT-PCR, with a correlation coefficient of 0.88 (P < 0.001). On the other hand, GI (n = 7) did not show a significant correlation (r = 0.27, P = 0.558). In terms of the major GI genotype, including more than 7 specimens, GI.3 (n = 7), GI.4 (n = 29), and GI.2 (n = 8) showed a good correlation (GI.3, r = 0.93, P = 0.002; GI.4, r = 0.92, P < 0.001; GI.2, r = 0.80, P = 0.017). On the contrary, GI.6 (n = 7) did not show a significant correlation (r = 0.69, P = 0.086).

Analytical sensitivity of the BLEIA. The analytical sensitivity of the BLEIA was determined by measuring the detectable virus concentration from 10-fold serially diluted NV-positive fecal supernatant. As shown in Table 2, the highest sensitivity of 7.0 × 10^4 copies/g of fecal sample was obtained from the sample of GI.3. On the other hand, the lowest sensitivity of 3.3 × 10^5 copies/g of fecal sample was obtained from the sample of GI.6. These results suggested that the analytical sensitivity of the BLEIA was estimated to be on average 7.1 × 10^5 copies/g of fecal sample, which was equivalent to 2.8 × 10^3 copies/test.

DISCUSSION

We developed an ultrasensitive and fully automated BLEIA for the detection of NV capsid antigen. In the sensitivity test with VLP, the assay demonstrated the sensitive detection of as little as 0.25 pg/ml capsid protein, which was equivalent to 1.5 × 10^4 VLPs/ml. This sensitivity is nearly equal to that of a previous BLEIA for prostate-specific antigen (27), indicating that we could fully educe the potential ability of the BLEIA as a high-sensitivity method for detection of protein antigen. In the dilution test with NV specimens, the analytical sensitivities of the BLEIA were confirmed to be 10^5 and 10^6 copies/g of fecal sample. According to the previous studies, various real-time RT-PCRs have the highest sensitivity, ranging from 10^4 to 10^5 copies/g of fecal sample, but they are not easy to perform and are time-consuming (11, 22). On the contrary, although several commercially available molecular methods have a little less sensitivity, ranging from 10^7 to 10^8 copies/g of fecal sample, they are more convenient and have also been widely used as a highly sensitive detection method for NV (7, 9, 21). Therefore, the BLEIA is expected to be used in various fields as well as commercially available molecular methods.

The BLEIA demonstrated broad reactivity against 14 genotypes of VLPs. However, the reactivity varied a little according to the genotypes. The difference between the highest reactivity (GI.1) and the lowest (GI.7) was about 20-fold. We assumed that the differences were largely attributable to the cross-reactivity of the antibodies employed in the assay. On the one hand, we also confirmed that VLPs produced from different batches demonstrated slightly different antigenicities (data not shown). Accordingly, it is suspected that the completeness of VLPs might moderately affect the evaluation with VLPs. Nevertheless, we believe that the approximately 20-fold difference in reactivities among the genotypes is not a serious problem in practical use because even widely used molecular methods have 10-fold different sensitivities depending on the genotype (7, 21). In the evaluation with NV specimens, the BLEIA detected all 10 genotypes and the index value was proportional to the viral loads, regardless of genotypes, indicating that the BLEIA can equally detect various genotypes of NV.

In order to obtain broad reactivity against various genotypes, we applied four mouse monoclonal antibodies, clones 107-5, v6-29, m9-110, and 212-60-5, to the BLEIA. When characterized with a VLP-immobilized ELISA, clone m9-110 recognized all 14 genotypes of
VLPs prepared for this study. Clone 107-5 recognized all 6 genotypes of genogroup I, and clone v6-29 recognized all 8 genotypes of genogroup II. On the other hand, clone 212-60-5 showed narrow reactivity to GII.1, GII.3, GII.6, and GII.12 (data not shown). Although we did not perform comprehensive epitope mapping of these antibodies, it is assumed that clone m9-110 is a GI and GII cross-reactive antibody and clones 107-5 and v6-29 are intragenogroup cross-reactive antibodies because it has been reported that there are some cross-reactive epitopes among the NV capsid proteins (15, 26, 28). Since clone 107-5 and clone v6-29 are employed as solid-phase antibodies, capsid antigens can be captured on magnetic particles via clone 107-5 or clone v6-29. On the other hand, because clones m9-110 and 212-60-5 are employed as labeled antibodies, broad reactivity of the labeled antibody is considered to be ensured by clone m9-110. Thus, we reason that the BLEIA can broadly detect various genotypes of NVs by using these antibodies. 

Compared with conventional immunological methods, such as ELISA and IC, the BLEIA has the following two advantages. First, the BLEIA is more sensitive. The previous studies showed that the sensitivities of ELISA and IC ranged from $10^6$ to $10^7$ copies/g of fecal sample (4, 14, 25, 29), while the sensitivities of the BLEIA ranged from $10^5$ to $10^6$ copies/g of fecal sample. Second, the BLEIA is more broadly reactive against various genotypes. In this study, the BLEIA could equally detect various genotypes of NV, while it is reported that some ELISA and IC tests showed significant differences in reactivities in genotypes. On the other hand, in terms of rapidity and simplicity, ELISA and IC are superior to the BLEIA. Especially, IC is the easiest assay to perform at the bedside because it takes at most about 20 min to complete and does not require special equipment and skills. Although ELISA takes more time than the BLEIA (at least 1.5 h), it is not necessary to use special equipment.

As described above, this method has great advantages against conventional detection methods for NVs. However, at least the following two issues should be investigated before the assay is widely employed in practical use. First, the positive reactions (values greater than 1.0) are highlighted in boldface. OR, over range; $-$, not tested.

### Table 2: Analytical sensitivity of the BLEIA

| Sample | Genotype | Virus copy no. of undiluted sample (copies/g of fecal sample) | Index value at dilution ratio of: | Estimate of analytical sensitivity (copies/g of fecal sample) |
|--------|----------|-------------------------------------------------------------|----------------------------------|----------------------------------------------------------|
| A      | GII.2    | $1.5 \times 10^6$                                          | 355.8                            | $7.5 \times 10^5$                                        |
| B      | GII.3    | $1.4 \times 10^{10}$                                       | OR                               | $7.0 \times 10^4$                                        |
| C      | GII.3    | $2.0 \times 10^7$                                          | 67.7                             | $1.0 \times 10^3$                                        |
| D      | GII.4    | $4.4 \times 10^5$                                          | 145.0                            | $2.2 \times 10^3$                                        |
| E      | GII.4    | $8.5 \times 10^6$                                          | 1,739.3                          | $4.3 \times 10^6$                                        |
| F      | GII.4    | $2.7 \times 10^8$                                          | 1,319.6                          | $1.4 \times 10^7$                                        |
| G      | GII.6    | $6.6 \times 10^8$                                          | 5,392.5                          | $3.3 \times 10^8$                                        |
| H      | GII.6    | $1.2 \times 10^9$                                          | 9,000.1                          | $6.0 \times 10^7$                                        |

$^a$ The NV-positive samples, which were quantified by real-time RT-PCR, were 10-fold serially diluted and measured by the BLEIA. The index values of diluted samples are indicated. The positive reactions (values greater than 1.0) are highlighted in boldface. OR, over range; $-$, not tested.
the BLEIA can detect other genotypes of NV that have not been examined yet. In this study, the BLEIA demonstrated broad reactivity to various NV genotypes, including genotypes 1, 2, 3, 4, 7, 8, and 12 in GI and genotypes 1, 2, 3, 4, 5, 6, 12, and 13 in GII, but there are other genotypes of NV that are often encountered in the clinical field (12). Therefore, we need to investigate further the reactivity to other genotypes of NV using many clinical samples. Second, no cross-reactions in fecal samples containing other bacteria and viruses should be confirmed. In this study, the BLEIA showed no cross-reactivity toward enteric bacteria, including Salmonella enterica serovar Enteritidis, Bacillus cereus, Campylobacter coli, Campylobacter jejuni, Citrobacter freundii, Clostridium perfringens, Enterococcus faecalis, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa, Vibrio parahaemolyticus, Candida albicans, Escherichia coli, and Bacteroides fragilis. Moreover, in a preliminary study, we have already confirmed that the BLEIA has no cross-reactivity toward other enteric viruses, such as sapovirus, rotavirus, adenovirus, and astrovirus (data not shown), but there are many other bacteria and viruses in individual fecal samples. Accordingly, further investigation of the cross-reactivity should be conducted by using many fecal samples containing other bacteria and viruses.

In conclusion, we have developed an ultrasensitive and fully automated bioluminescent enzyme immunoassay for NV capsid antigen. Because the BLEIA has high-sensitivity, broad reactivity against various NV genotypes, and good reproducibility, it is potentially useful for the rapid diagnosis of NV in epidemic and sporadic gastroenteritis. Moreover, it is now well known that NV outbreaks are often caused by asymptomatic carriers in semiclosed communities because they shed virus through feces over several days (1). Therefore, for the sake of public health, it is very important to screen asymptomatic individuals for NV infection periodically by using a sensitive and rapid diagnostic method. Because the BLEIA for NVs is highly sensitive and has high throughput, it will be a powerful tool not only for the daily routine diagnosis of NV, but also for the prevention of NV outbreaks.

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