Establishment and characterization of noro-VLP measurement by digital ELISA

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Abstract

Highly sensitive viral analytical techniques are essential tools for preventing the spread of infections. In this study, we established a digital enzyme-linked immunosorbent assay (ELISA) system to quantify norovirus proteins with high sensitivity. We used norovirus-like particles (noro-VLPs) as a surrogate for norovirus and constructed two digital ELISA systems using two different antibody pairs. The quantitative performance of the noro-VLP measurement using each digital ELISA system was evaluated. Both assay systems exhibited high sensitivity, good linearity, and high stability. The first system exhibited a limit of detection (LOD) of 87 pg/mL, correlation coefficient (R^2) of 0.9984, inter-assay variation of 5.5 %, and intra-assay variation of 5.2 %. The second system exhibited an LOD of 19 pg/mL, R^2 of 0.9984, inter-assay variation of 4.5 %, and intra-assay variation of 2.5 %. Comparison of the two systems using the same calibrant for unpurified and fractionated noro-VLPs revealed that the quantitative values for unpurified noro-VLPs were the same, whereas those for fractionated noro-VLPs were dramatically different. Our findings indicate that the reactivity to various components in the noro-VLP solution was altered depending on the different antibodies. Furthermore, our study highlights the importance of using appropriate calibrants, which contain the same ratio of components as the noro-VLP analyte, to afford accurate measurements.

Introduction

Norovirus is a non-enveloped virus that infects humans; it causes nausea, vomiting, abdominal pain, fever, chills and diarrhea.[1] Numerous cases of norovirus infection are reported annually, leading to a significant number of deaths.[2] The main routes of norovirus infection are through human vomit and feces, or by eating food contaminated during the production or cooking procedures.[3,4,5] Although many people have been affected by this disease, antiviral agents and vaccines are still in the development stage, with supportive care being the primary treatment.[6] Therefore, surveillance

and disinfection are currently the only effective methods for preventing the spread of norovirus infections.[7,8]

Highly sensitive viral measurement methods are important and effective in preventing the spread of infections. For example, testing of food, food production environments, and hospitals can significantly contribute to reducing the risk of norovirus infection. Polymerase chain reaction (PCR) is commonly used to detect noroviruses and is adopted in ISO 15216.[9] PCR targets noroviral RNA and detects it by gene amplification. However, PCR is not sufficient for accurate measurements of noroviruses because RNA measurements alone cannot distinguish between infectious and non-infectious viruses or free unencapsidated RNA in a sample.[10] To detect infectious viruses with high sensitivity, viral RNA, proteins, and particles should be measured and combined for a multifaceted evaluation. Enzyme-linked immunosorbent assay (ELISA) is a conventional method to measure proteins, however, it is less sensitive than PCR for viral measurements.[11] Therefore, highly sensitive protein measurement techniques must be developed for accurate viral measurements.

Herein, to increase the sensitivity of norovirus measurements using immunoassays, we focused on digital ELISA. In digital ELISA, the antigen–antibody reaction is performed on microbeads; the microbead–antibody–antigen–antibody-β D galactosidase (SBG) complex is dispersed into the microwells. The amount of target protein is measured by detection in each microwell and counting the number of positive and negative wells.[12,13] Digital ELISA systems are approximately 1000 times more sensitive than conventional ELISA.[12,14]

In this study, we established a digital ELISA system to quantify norovirus proteins with high sensitivity. Although several studies on the development of culture systems for noroviruses have been reported, challenges remain and standard methods have not been established.[15] In addition, experiments using infectious noroviruses need to be performed in biohazard safety facilities. Herein, Norovirus virus-like particles (noro-VLPs) were used as targets for our measurements. These particles were assembled from 180 copies of recombinant VP1 of norovirus expressed in cultured cells.[16] Noro-VLPs are morphologically and antigenically similar to noroviruses; however, they are not infectious because noro-VLPs do not contain nucleic acids and can be utilized in facilities with low biosafety levels. Therefore, noro-VLPs are used as an alternative to noroviruses in analytical method development and vaccine development.[17,18] First, the optimal antibody pairs for the noro-VLP assay were screened using conventional plate ELISA, and an ultrasensitive quantitative digital ELISA system was established using these antibodies. The linearity, sensitivity, and robustness of the assay were evaluated under established assay conditions. In addition, the effect of unpurified or fractionated VLP on the quantitative results was evaluated using unpurified VLP as a calibrant.

Experimental

Reagents

Anti-norovirus protein antibodies (ab125151, ab125039, ab167024, and ab252725; abbreviated as a51, a39, a24, and a25, respectively) were purchased from Abcam Plc. (Cambridge, UK). Recombinant Norovirus GII.4VP1 virus-like particles were purchased from Abcam (Cambridge, UK). Phosphate-buffered saline (PBS) tablets were purchased from Takara Bio, Inc. (T9181, Japan). A protein mixture containing thyroglobulin, IgG, bovine serum albumin, myoglobin, and uracil (BEH200 SEC Protein Standard Mix) was purchased from Waters Corporation (product no.186006518, MA, USA). The human serum albumin used was a certified reference material developed in our laboratory (NMIJ CRM 6202-a).

Digital ELISA

The digital ELISA systems were established using the Simoa Homebrew Assay Development Kit (Quanterix, MA, USA) and Simoa SR-X (Quanterix) following the manufacturer's instructions. Briefly, the carboxylated capture beads were activated using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (Thermo Fisher Scientific, Waltham, MA, USA) and were conjugated with the capture antibody. The detection antibody was biotinylated using NHS-PEG4-Biotin (Thermo Fisher Scientific). Beads with capture antibodies and biotinylated detection antibodies were purified and stored at 4 °C until further use.

SR-X measurements were performed in accordance with the manufacturer's instructions. All the calibrants and measurement samples were prepared using low-protein-binding pipette tips and tubes. The noro-VLP sample was diluted with the sample dilution solution using the Simoa Homebrew Assay Development Kit. Briefly, 25 µL of beads (2×10^7 particles/mL) with capture antibodies and 20 µL of 0.7 µg/mL biotinylated detection antibodies were added to the measurement sample (100 µL). SBG (5 pM) was reacted with the bead–antibody–antigen antibody, mixed using a Simoa microplate shaker (Quanterix), and washed with a 405 TS BioTek microplate washer (Agilent, CA, USA). The reacted bead complexes were placed in the SR-X and analyzed. The measurement result of SR-X is shown as the average enzymes per bead (AEB). The AEB was calculated using the following formula:

$$AEB_{digital} = -\ln(1 - f_{on}) \cdots (f_{on} < 0.7)$$
$$AEB_{analog} = (f_{on} \times I_{bead})/I_{signal} \cdots (f_{on} > 0.7)$$

where f_{on} is ratio of beads with enzyme activity to total beads, I_{bead} is average fluorescence intensity per bead, I_{singal} is average fluorescence intensity per enzyme. SR-X can quantify a wide concentration range using two analysis modes: digital analysis mode and analog analysis mode. The measurements in this study were performed only in the digital analysis mode, which is reported suitable for measuring low-concentration samples.[14]

Sample Preparation

For the purification of noro-VLPs, size-exclusion chromatography was performed using a Nexera X2 system (Shimadzu, Japan). The monitoring wavelength was set at 215 and 280 nm. The column temperature was maintained at 30 °C. The mobile phase comprised PBS. Protein mixtures and human serum albumin were used as the molecular weight markers. Each fraction, including the two peaks observed in the UV chromatogram, was manually collected. One fraction contained the particle component with a retention time faster than that of thyroglobulin (660 kDa), and the other fraction contained the VP1 monomeric component with a retention time equivalent to that of human serum albumin (66 kDa). Each fraction was stored at −80 °C until further use.

Results and discussion

Suitable antibody pairs were selected to construct a noro-VLP assay system using digital ELISA. a51, a39, a24, and a25 were tested as capture and detection antibodies for screening 16 antibody pairs. Conventional plate ELISA was performed for primary screening and digital ELISA was performed for secondary screening. The digital ELISA system was constructed for the two antibody pairs a51–a24 and a39–a24 (in the order of capture–detection antibodies), and commercial noro-VLPs were used to prepare calibration solutions ranging from 2 to 10 pg/mL, the concentrations of which were calculated according to the manufacturer's instructions. Figure 1 shows the calibration curves for noro-VLPs for each antibody pair. The assay using the a51–a24 antibody pair exhibited good linearity, with a correlation coefficient (R^2) of 0.9984. The limit of detection limit of detection (LOD) (3 SD blank/slope) and limit of quantification (LOQ) (10 SD blank/slope) were calculated as 87 and 290 pg/mL, respectively. Furthermore, the assay using the a39–a24 antibody pair showed good linearity, with an R^2 of 0.9984, whereas the LOD and LOQ were 19 and 62 pg/mL, respectively. In general, the LOD of conventional ELISA systems is in the ng/mL concentration level.[19,20] Therefore, the digital ELISA system established in this study is approximately 150 times more sensitive than conventional ELISA systems.

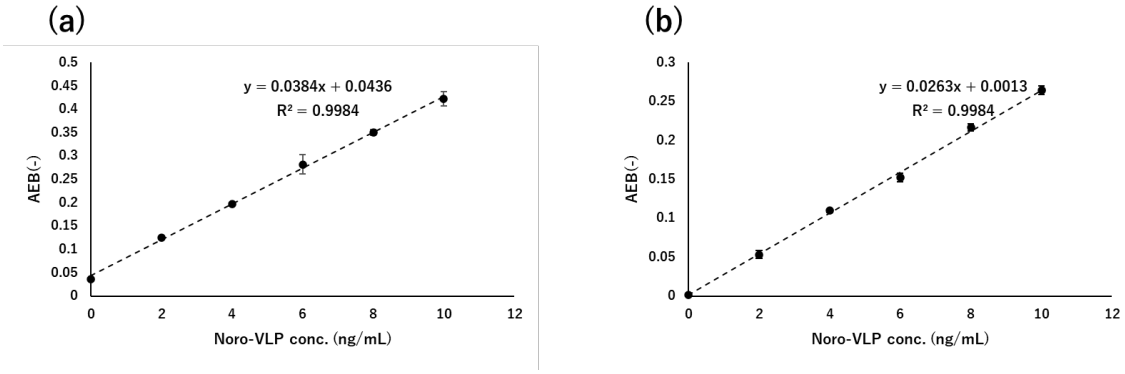

Figure 1. Calibration curves of noro-VLP analyses via digital ELISA. (a) Calibration curve using the a51–a24 antibody pair. (b) Calibration curve using the a39–a24 antibody pair. Data are presented as the mean ± SD (n = 3).

Assessment of the robustness of a measurement is important for method validation.[21] To evaluate the robustness of the developed digital ELISA system for noro-VLPs, the inter- and intra-assay variations were analyzed. Noro-VLP solutions were diluted to 5 ng/mL, which is the concentration at the middle of the dynamic range, and were analyzed. Their concentrations were calculated using the calibration curves. The measurements were performed independently in triplicate for 3 days. From these results, inter- and intra-assay variations were calculated using one-way analysis of variance (ANOVA). Table 1 shows each variation in noro-VLP measurements using the two assay systems. The total average was 5.20 ng/mL for the assay using the a51–a24 antibody pair, whereas the inter- and intra-assay variations were 5.5 % and 5.2 %, respectively. In contrast, the total mean was 5.02 ng/mL for the assay using the a39–a24 antibody pair, whereas the inter- and intra-assay variations were 4.5 % and 2.5 %, respectively. Although the variability of digital ELISA is still not fully understood, for conventional ELISA systems, it has been reported that an assay is stable when the inter- and intra-assay differences are less than 15 % and 10 %, respectively.[22,23,24] In our previous study regarding the measurement of influenza virus recombinant proteins (nucleoprotein [NP] and hemagglutinin [HA]) via digital ELISA, we reported intra- and inter-assay variations of 5.8 % and 5.2 % for NP and 2.5 % and 11.1 % for HA, respectively.[14] Furthermore, the intra- and inter-assay variations of the NP influenza virus particles were 20.1 % and 12.1 %, respectively, whereas those of the HA influenza virus particles were 0.0 % and 9.1 %, respectively. Therefore, in this study, we concluded that the variations in the noro-VLP measurements were more stable compared to those of the influenza virus particles, and were comparable to those of the influenza virus recombinant protein measurements. This is possibly attributed to noro-VLPs being measured without the lysis process, whereas the influenza measurement was performed after the virus was lysed. In this study,

the inter- and intra-assay variations for both antibody pairs were approximately 5 %. The total variation for both antibody pairs was less than 10 %, indicating that the assay was highly robust.

Table 1. Analytical results of the noro-VLP samples using the a51-a24 and a39-a24 antibody pairs. The variations were calculated via one-way ANOVA.

Antibody pair	a51-a24			a39-a24		
Assay	1	2	3	1	2	3
Measured	5.37	5.25	5.04	5.36	4.95	5.08
value (ng/mL)	5.12	5.00	5.17	5.02	4.97	4.75
	6.46	4.68	4.69	5.57	4.69	4.77
Total average (ng/mL)		5.20			5.02	
Variation (%)						
inter-assay		5.5			4.5	
intra-assay		5.2			2.5	
total		7.5			5.1	

A noro-VLP was assembled from 180 copies of VP1 with a T = 3 icosahedral symmetry. However, noro-VLPs are known to coexist with particles of other sizes, such as T = 1, 60-copies of VP1 and T = 4, 240-copies of VP1.[25,26] To investigate the effect of different noro-VLP components on the assay, unpurified noro-VLP samples, particle fraction samples, and VP1 fraction samples fractionated after separation via chromatography were quantified using the two digital ELISA systems. The concentration of each sample was calculated from the calibration curve with unpurified noro-VLPs and normalized to the average of the a51–a24 antibody pair measurement results. Figure 2 shows the measured values for each sample. The normalized values of the unpurified noro-VLPs measured using the a51–a24 and a39–a24 antibody pairs were comparable (Figure 2a). In contrast, the normalized values of the particle fraction sample and the VP1 fraction sample measured using the a39–a24 antibody pair were low compared to those of the a51–a24 antibody pair (Figure 2b, c). This is possibly due to differences in the reactivity of the two antibody pairs to each component of the noro-VLPs. In this study, the unpurified noro-VLP sample used as the calibrant contained a mixture of various components of noro-VLP, whereas the particle and VP1 fraction samples only contained certain components of noro-VLP. If the ratio of the noro-VLP components in the calibrant was the same as that in the measurement sample, the assay would afford an accurate measurement. In other words, if the ratio of each noro-VLP component differs from the calibrant in the actual sample, there is a risk that the measured value will be biased from the true value. Such a conclusion was confirmed by our results, which showed that the reactivity of the

antibodies differed between the fraction samples. Therefore, it is recommended to prepare and utilize a calibrant corresponding to each VLP component when conducting norovirus sample analysis using noro-VLPs as calibrants.

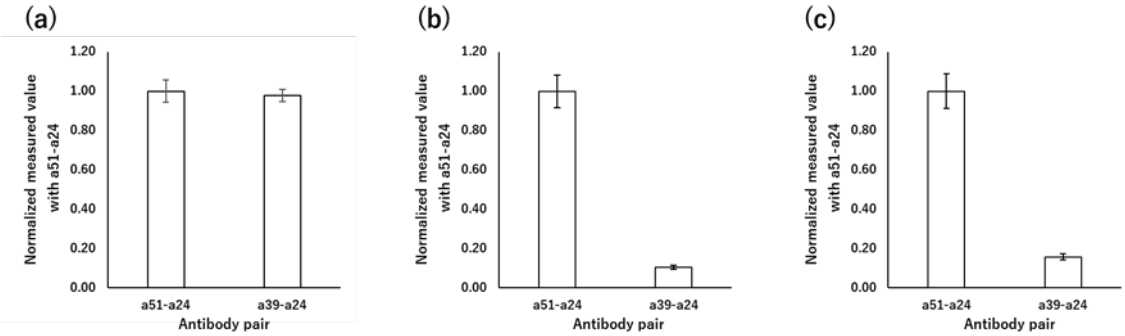

Figure 2. Measured values of (a) unpurified noro-VLP, (b) particle fraction, and (c) VP1 fraction. Each measured value was normalized with the average measured value using the a51–a24 antibody pair in each sample.

Conclusions

In this study, a noro-VLP assay system using digital ELISA was constructed and quantitatively evaluated. Good linearity and a high sensitivity in the pg/mL levels were achieved using two different antibody pairs. In addition, analysis of samples containing 5 ng/mL noro-VLP confirmed the high robustness of the method, indicating that consistent measurements are possible. Furthermore, we demonstrated that each noro-VLP component in the solution reacted differently with the antibodies. The antigenicity of conventional ELISA has been shown to differ depending on the VLP particle size.[25] In this study, using two different antibody pairs, we confirmed that antigenicity differs depending on the VLP composition in digital ELISA too. Furthermore, the non-particle VP1 exhibited different antigenicities. In immunoassays using noro-VLPs, differences in the composition of each noro-VLP result in bias in the measurement results. To solve this problem, a technique for evaluating the content of each component in a noro-VLP solution is required. In addition, to use noro-VLP as a calibrant in actual sample analysis for noroviruses, it is important to develop a reference material that evaluates the ratio of each component of noro-VLP, or a purified material that contains complete noro-VLP alone and is evaluated for purity and concentration.

Author Contributions

Takema Hasegawa: conceptualization, data curation, formal analysis, investigation, methodology, visualization, writing – original draft. Yuriko Adachi: investigation, methodology, writing – review & editing. Kazumi Saikusa: investigation, methodology, writing – review & editing. Megumi Kato:

conceptualization, funding acquisition, methodology, project administration, supervision, writing – review & editing.

Conflicts of interest

There are no conflicts to declare.

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