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A Luciferase Immunoprecipitation System (LIPS) Assay for Profiling Human Norovirus Antibodies

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Abstract

A luciferase immunoprecipitation systems (LIPS) assay was developed to define the antigenic specificity and titer of antibodies directed against human norovirus (HuNoV). Recombinant proteins, expressed by plasmid constructs encoding *Renilla* luciferase (Ruc) fused to the full-length HuNoV major capsid protein (VP1) (Ruc-antigen), were generated for ten HuNoV strains. In addition, subdomain constructs Ruc-Shell (S) and Ruc-Protruding (P) were engineered for a representative GII.4 norovirus (strain GII.4/2006b). The LIPS assay measured antibody levels in a well-defined panel of HuNoV-specific sera, and the results were compared to an ELISA standard. In hyperimmune sera, the LIPS produced titers similar to or higher than those measured by the ELISA of HuNoV-specific antibodies. The specificity of antibodies in various sera was profiled by LIPS with a panel of diverse Ruc-antigens containing full-length HuNoV VP1 proteins or VP1 subdomains, and the assay detected both specific and cross-reactive antibodies. Competition assays, in which antibodies were pre-incubated with one or more intact VLPs representing different genotypes, proved useful in further assessment of the antibody specificity detected by LIPS in complex polyclonal sera. The profiling of HuNoV-specific antibodies in the high-throughput LIPS format may prove useful in defining the strength or specificity of the adaptive immune response following natural infection or vaccination.

Keywords

Human norovirus; LIPS assay; ELISA; Serum antibody

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1. INTRODUCTION

Human norovirus (HuNoV), a major etiologic agent of acute gastroenteritis, contributes to an estimated 70,000 to 200,000 deaths in children in developing countries (Patel et al. 2008, Lanata et al. 2013). HuNoV is also a major economic burden in developed countries, as healthcare costs for medically attended HuNoV cases are approximately \$273 million per year in the United States (Payne et al. 2013).

Noroviruses are classified in the family *Caliciviridae* (genus *Norovirus*), a group of small, non-enveloped, icosahedral viruses with a single-stranded positive-sense RNA genome (Green et al. 2000). The norovirus genomic RNA is approximately 7.7 kb in length and its genome is organized into three open reading frames (ORFs). ORF1 encodes the nonstructural proteins, ORF2 encodes the major capsid protein VP1, and ORF3 encodes the minor capsid protein VP2 (Bertolotti-Ciarlet et al. 2003, Glass et al. 2000). The 180 copies of VP1 are assembled into an icosahedral protein shell that encloses the virus RNA genome. VP1 contains two domains linked by a flexible hinge: the shell (S) domain and the protruding (P) domain, the latter of which is divided into the P1 and P2 subdomains (Prasad et al. 1999).

The *Norovirus* genus is comprised of seven genogroups (GI–GVII), with over 40 diverse genotypes defined by sequences encoding the VP1 protein and RNA-dependent RNA polymerase (RdRp) (Kroneman et al. 2013, Vinje 2015). GI and GII are the most commonly detected genogroups in human outbreaks (11% and 89%, respectively) (Vega et al. 2014). The diversity of strains within these genogroups, specifically those of genotype GII.4, has been linked to a high nucleotide substitution rate of approximately 4.3×10^{-3} substitutions/site/year in the HuNoV major capsid protein gene (Bok et al. 2009, Bull et al. 2010, Duffy, Shackelton, and Holmes 2008). These mutations in the HuNoV genome contribute to the virus' antigenic diversity, particularly in the surface-exposed P2 subdomain, which contains diverse epitopes that may be under strong selective pressure to escape herd immunity (Lindesmith et al. 2012, Lindesmith et al. 2008).

Assessing neutralizing antibodies and their role in protection and immunity has been difficult in the absence of fully permissive cell culture systems for HuNoV infection and replication. Studies have shown that the HuNoV major capsid protein binds to HBGAs in a strain-specific manner (Singh, Leuthold, and Hansman 2015, Hutson et al. 2002, Hutson et al. 2003, Parra et al. 2012), which may influence the host's susceptibility to virus infection (Hutson et al. 2002). Blocking antibodies that inhibit virion binding to HBGAs have been considered a correlate of protection (Atmar et al. 2015), and understanding the adaptive immune response to HuNoV has been an important goal in vaccine development.

The Luciferase Immunoprecipitation Systems (LIPS) assay is a liquid phase immunoassay allowing high-throughput serological screening of antigen-specific antibodies. The immunoassay involves quantitating serum antibodies by measuring luminescence emitted by the reporter enzyme *Renilla* luciferase (Ruc) fused to an antigen of interest, expressed by the pRen2 (pRuc) vector in mammalian cells. The Ruc-antigen fusion protein is recognized by antigen-specific antibodies, and antigen-antibody complexes are captured by protein A/G

beads which recognize the Fc region of the IgG antibody (Burbelo et al. 2009). In this study, a LIPS assay was developed to evaluate the titer and specificity of serum antibodies against several HuNoV strains. We show that this assay performs well in profiling the adaptive immune response following immunization.

2. MATERIAL AND METHODS

2.1 Serum samples

2.1.1 Minipig sera—Serum samples were collected from two conventionally raised Gottingen miniature pigs (minipigs) (Marshall BioResources, North Rose, NY) immunized with norovirus VLPs following failure to infect them with human norovirus by the oral and intravenous routes. A mock-immunized minipig served as the control. The VLP immunogens were adsorbed by Alhydrogel as an adjuvant as previously described (Bok et al. 2011), and administered intramuscularly three times at two-week intervals. A booster dose was given five weeks after the third immunization. The HuNoV strain origin of the VLPs and their dosages are provided in Table 1. The tested sera were collected at post-inoculation week (PIW) 0, 2, 4, 7, 11, and 15. Serum samples were stored at -20°C and thawed prior to LIPS analysis. All minipig studies were conducted at the NIH, Bethesda, Maryland under an animal protocol (LID13) approved by the NIAID Division of Intramural Research Animal Care and Use Committee.

2.1.2 Guinea pig sera—Hyperimmune sera raised in guinea pigs against GII.4/2004 VLPs were produced as previously described (Parra et al. 2012) under an animal protocol (LID 73) approved by the NIAID Division of Intramural Research Animal Care and Use Committee. Production of guinea pig hyperimmune sera against GII.4/2006b VLPs was carried out by Rockland Immunochemicals Inc. (Limerick, PA), and approved by the Institutional Animal Care and Use Committee put in place by Rockland facilities (licenses and assurance numbers: USDA #23-R-134; NIH BPA #00008695; NIH Assurance OPRR #A4062-01).

2.2 Construction of expression plasmids

ORF2 sequences encoding the complete VP1 protein, or its domains S and P, from various HuNoV strains were subcloned into mammalian expression vector pRen2 (pRuc) (Burbelo et al. 2009). Plasmid vectors and/or baculovirus DNA that express VLPs in the baculovirus system were used to amplify VP1 and S- or P-domain sequences for the pRuc constructs (Esseili, Wang, and Saif 2012, Leite et al. 1996, Green et al. 1993, Bok et al. 2009, Green et al. 1997, Kocher et al. 2014, Lew et al. 1994, Parra and Green 2014, Jiang et al. 1992). The recombinant baculovirus carrying the genes for the major capsid protein of a GII.4/2006b strain (GenBank #KC990829), collected from a child with NoV gastroenteritis in 2008, was generated using the BaculoDirect baculovirus expression system (Thermo Fisher Scientific, Waltham, MA) as previously described (Kocher et al. 2014, Jiang et al. 1992).

The following HuNoV strains were used to generate pRuc constructs (Table 2): Hu/NoV/GI.1/Norwalk/1968/US, Hu/NoV/GI.5/SzUG1/1997–99/JP, Hu/NoV/GI.6/HSS3/1997/DE, Hu/NoV/GII.1/Hawaii/1971/US, Hu/NoV/GII.2/SnowMountain/1976/US, Hu/GII.3/

CHDC2005/1975/US, Hu/NoV/GII.4/MD145-12/1987/US, Hu/GII.6/BethesdaD1/2012/US, Hu/NoV/GIV.1/SaintCloud624/1998/US, and Hu/GII.42006b/092895/2008/US.

The primers employed in PCR incorporated a unique restriction site (*Hind*III, *Bam*HI, *Xba*I, *Xho*I, or *Not*I, shown as underlined) and a stop codon (shown in **bold**) into the amplified HuNoV ORF2 gene (Table 2). Gel-purified PCR fragments from each region were digested with their corresponding restriction enzyme and ligated into the double digested (*Hind*III-*Xho*I, *Bam*HI-*Not*I, *Xba*I-*Not*I, or *Xho*I-*Not*I) pRuc vector. The ligation mixtures were used to transform One Shot® OmniMAX™ 2 T1^R Chemically Competent *E. coli* cells (Thermo Fisher Scientific), and transformed cells were plated on LB agar plates with 50 µg/mL kanamycin at 37°C overnight. Clones were screened using sequencing analysis and the desired plasmids were selected for amplification. The resulting constructs contained sequences of VP1, S-, or P-domains fused to the C-terminus of the Ruc protein, and were designated pRuc-VP1, pRuc-S, or pRuc-P, respectively.

2.3 Transfection of COS1 cells

Using Lipofectamine LTX with Plus Reagent (Thermo Fisher Scientific), COS1 cells were transfected with each individual plasmid construct, according to the manufacturer's protocol. Briefly, cells were plated at 1×10^6 cells per well of a 6-well cell culture plate (Corning Inc., Corning, NY) and grown in 2 mL of DMEM with 4.5 g/L of glucose (Lonza, Walkersville, MD) supplemented with 10% FBS, 100 U/mL of penicillin-streptomycin, and 292 mg/L (or 2 mM) of L-glutamine. When cell monolayers were approximately 95% confluent, cells were transfected with pRuc constructs or empty pRuc vector as a control.

Optimum transfection conditions for reporter enzyme activity, or highest RLUs produced from crude cell lysates, were assessed using checkerboard titrations of Lipofectamine LTX transfection reagent against DNA concentration. The optimum transfection conditions per well of a 6-well plate were determined as follows: 4 µg pRuc-VP1 DNA and 14 µL transfection reagent, 5 µg pRuc-S DNA and 11.25 µL transfection reagent, 2.5 µg pRuc-P DNA and 11.25 µL transfection reagent, or 5 µg pRuc DNA and 11.25 µL transfection reagent.

2.4 Confirming Ruc-antigen expression

2.4.1 Western blot analysis—Transfected cells were lysed 48 hrs post-transfection with 2 x Tris-glycine SDS sample buffer (Thermo Fisher Scientific). Lysates were sonicated and heated with 2.5% (v/v) 2-mercaptoethanol for 7 min at 96°C. Denatured proteins were separated by SDS-PAGE in 4–20% Tris-glycine gels (Thermo Fisher Scientific). Using the iBlot Dry Blotting System (Thermo Fisher Scientific), proteins were electroblotted onto a nitrocellulose membrane. The membranes were blocked with 1% normal goat serum (Kirkegaard & Perry Laboratories, Gaithersburg, MD) in Blotto (PBS with 5% nonfat dry milk) for 1 hr at room temperature (RT). Rabbit anti-Renilla Luciferase Polyclonal Antibody (Thermo Fisher Scientific), or mouse anti-beta actin monoclonal antibody (Sigma Aldrich, St. Louis, MO) was added to the membranes at a 1:1000 or 1:5000 dilution, respectively, and incubation with antibodies was carried out overnight at 4°C. Bound primary antibodies were detected by 1 hr incubation of the membranes with goat anti-rabbit or anti-mouse IgG (H

+L)-peroxidase labeled secondary antibody (1:2000 dilution) and treatment of membranes with SuperSignal West Pico Substrate (Thermo Fisher Scientific). Membranes were washed three times with PBS-0.1% Tween 20 (PBST) between each incubation step.

2.4.2 Indirect immunofluorescence assay—48 hrs post-transfection, rabbit anti-*Renilla* Luciferase polyclonal antibody, hyperimmune serum raised against a GII.4/2006b norovirus variant, or a monoclonal antibody (mAb) specific to a conformational epitope on the P-domain (Parra et al. 2012) was diluted 1:100 in PBS containing 1% normal goat serum (Kirkegaard & Perry Laboratories), and incubated with methanol-fixed cell monolayers overnight at 4°C. Following three washes with PBS, bound antibody-antigens were detected using Alexa Fluor®-conjugated goat anti-guinea pig, anti-rabbit, or anti-mouse IgG antibodies (Thermo Fisher Scientific) diluted 1:200 in PBS. Fluorescence was detected using a Leica DMI4000 B microscope (Leica Microsystems, Wetzlar, Germany). The corresponding images were captured with a QImaging Retiga-2000R camera (QImaging, Surrey, BC, Canada) and processed using iVision 4.0.14 software (BioVision Technologies, Exton, PA).

2.5 Serological immunoassays

2.5.1 LIPS assay—Cells expressing Ruc-antigen proteins were lysed using 1.4 mL of lysis buffer A (50 mM Tris, pH 7.5, 100 mM NaCl, 5 mM MgCl₂, 1% Triton X-100, 50% glycerol, and 1x Halt protease inhibitor cocktail [Thermo Fisher Scientific]) per 6-well plate, or approximately 235 µL per well, as previously described (Burbelo et al. 2009). Supernatant that was collected from crude lysates and clarified by centrifugation at 12,000 ×g was stored at –80°C. The lysates were thawed on ice prior to LIPS analyses.

Serum samples were processed in a 96-well format in duplicates at RT as previously described (Burbelo et al. 2009). Hyperimmune guinea pig sera containing known HuNoV-specific antibodies were used as a positive control, and pre-immunization sera were used as a negative control. Briefly, a master mix plate of serum samples diluted 1:10 in buffer A (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 5 mM MgCl₂, 1% Triton X-100) was made. The experimental measurements were carried out in two research laboratories where two different luminometers with different instrument protocols were used. Lysates of COS1 cells expressing 10⁷ relative light units (RLUs) of Ruc-antigen were used for each serum sample in order to standardize the amount of input antigen utilized for each test. To calculate the amount of lysate to use for the assays, RLUs of each Ruc-antigen protein were first measured in a Berthold LB 960 Centro microplate luminometer (Berthold Technologies, Bad Wilbad, Germany) or a Synergy Neo2 Multi-Mode Reader (BioTek Instruments, Inc., Winooski, VT) with a 96-well, white polystyrene, and opaque bottom microtiter plate (Thermo Fisher Scientific) that measured RLU per µL of cell lysate. Then, in a 96-well vinyl microtiter U-bottom plate, antigen and antibodies were incubated by adding 40 µL of buffer A, 10 µL of the 1:10 diluted sera, and 10⁷ RLUs of Ruc-antigen diluted into 50 µL buffer A. For the competitive LIPS assay, 2 µg of VLPs were added to the serum mixture overnight at 4°C prior to the addition of Ruc-antigen. 100 µL of the antibody and Ruc-antigen (or VLP, antibody, and Ruc-antigen) reaction were incubated for 1 hr on a rotary shaker, and then the mixture was transferred from the 96-well vinyl microtiter U-bottom plate to a 96-well filter

HTS plate (EMD Millipore, Billerica, MA) coated with 5 μ L of Protein A/G Plus UltraLink Resin beads (Thermo Fisher Scientific) diluted as a 30% suspension in PBS. After a 1 hr incubation, filter plates were washed on a vacuum manifold (Bio-Rad Laboratories, Hercules, CA) using 200 μ L/well of buffer A once, 100 μ L/well of buffer A 7 times, and 100 μ L/well of PBS 3 times. After the final wash, RLUs were measured with the microplate luminometer using the Renilla Luciferase assay substrate (Promega). The settings of the Berthold luminometer included injection of 50 μ L of substrate, shaking the plate for 2 sec, and using a 5 sec integration read. Settings of the Synergy luminometer included injection of 50 μ L of substrate, shaking the plate for 2 sec, and using a 0.2 sec integration read. RLU values were corrected either by dividing the average RLUs of tested sera by the average background RLU, thus presenting mean fold rise (MFR), or subtracting the average background RLU from that of the tested sera, thus presenting average RLUs. MFR was used for experiments comparing multiple or different serum samples, and average RLUs were used for titration assays for optimization or competitive inhibition assays. Background was determined as incubated protein A/G beads, pre-immunization sera, and the respective antigen. Data represent the geometric mean of two or more independent experiments. Cutoff values were determined as the MFR or RLUs measured in sera from a mock-immunized minipig, plus three standard deviations.

2.5.2 ELISA titrations—An indirect, IgG-specific ELISA to measure antibody titers in hyperimmune sera, raised in guinea pigs against GII.4/2006b or GII.4/MD2004, was performed as previously described (Parra et al. 2012). Briefly, 96-well polystyrene microtiter plates were coated overnight at 4°C either with PBS (mock antigen) or with 100 ng of purified HuNoV VLPs (GII.4/2006b) that were used to generate the anti-GII.4/2006b hyperimmune sera. VLPs were produced using the BaculoDirect Baculovirus Expression System (Thermo Fisher Scientific) expressing C-terminal fused His-tagged recombinant VP1 proteins, using previously described methods (Jiang et al. 1992). After coating, wells were washed with PBST, then blocked with Blotto for 1 hr at RT. Four-fold dilutions of hyperimmune or pooled unimmunized sera (from an initial 1:10 dilution in PBS) were added to the mock- or VLP-coated wells and incubated for 1 hr at RT. After incubation, the wells were washed, and goat anti-guinea pig IgG conjugated to horseradish peroxidase (HRP) (1:2000 dilution; Kirkegaard & Perry Laboratories) was added. Following 1 hr incubation at RT and further washing, the bound secondary antibodies were detected using ABTS substrate (Kirkegaard & Perry Laboratories). The data represent the geometric mean of two or more independent experiments.

2.5.3 LIPS assay titrations—The LIPS assay also measured HuNoV-specific antibody titers of hyperimmune guinea pig sera raised against a GII.4/2006b or GII.4/MD2004 variant. First, hyperimmune or pooled unimmunized sera were titrated four-fold (initial serum dilution of 1:5 in a final volume of 50 μ L) in a 96-well vinyl microtiter U-bottom plate. Then, equal volume of Ruc-antigen (10^7 RLU of lysate in 50 μ L buffer A) was added to each well, further diluting the sera by a factor of 2 (total volume of 100 μ L). The rest of the LIPS assay protocol was followed as described in Section 2.5.1.

2.6 Statistical analyses

Graphs were generated using GraphPad Prism 7.0, and Tukey's multiple comparisons test in ordinary one-way ANOVA was used to analyze statistically significant differences ($p < 0.05$) among data.

3. RESULTS

3.1 Expression of Ruc-antigen fusion proteins in transfected cells

A panel of Ruc-VP1, -P, or -S fusion proteins was constructed for the LIPS assay (Table 2), which is based on the transient expression of active Ruc fused to an antigen of interest in mammalian cells. To verify expression of Ruc and Ruc-antigens, western blot analysis of cell lysates prepared 48 hrs following transfection of COS1 cells with the GII.4/2006b norovirus pRuc constructs (Table 2) was first performed. Probing with anti-Ruc antibodies showed the presence of the expected bands corresponding to proteins with molecular masses of 36 (Ruc), 96 (Ruc-VP1), 64 (Ruc-S), or 66 (Ruc-P) kDa (Fig. 1A, lanes 1, 3, 4, and 5, respectively). Ruc-related proteins were not detected in mock transfected, control cells (Fig. 1A, lane 2). Western blot analysis of the remaining full-length VP1 constructs (Fig 1B, lanes 1 to 9) identified a strong signal for each Ruc-fusion protein at the expected size, with the exception of GII.2 Ruc-VP1 (Fig 1B, lane 5), which reacted weakly with the anti-Ruc antibodies. Anti- β actin was used as a loading control. The identity of smaller products in some lysates (Fig 1A, lanes 4 and 5; Fig. 1B, lanes 1, 2, 4, and 6) was not determined, but was likely due to proteolytic cleavage of the fusion protein by cellular proteases.

We next confirmed the expression of Ruc and Ruc-antigens in transfected cells by immunofluorescent staining assay (IFA), which detects both linear and conformational epitopes (Parra et al. 2012). Positive staining of the GII.4/2006b Ruc-antigen constructs (VP1, S, and P) expressed in transfected cells was detected with homologous anti-VLP hyperimmune serum, indicating that antibodies against both the S- and P-domains were represented in the serum (Fig. 1C). Ruc expression was detected in cells for each of the GII.4/2006 Ruc-antigen constructs.

We previously reported the isolation of a mAb (A3) that recognized a conformational epitope involving amino acid residues 294 and 295 of the GII.4/MD145 norovirus P-domain (Parra et al. 2012). These results were supported using IFA for the Ruc-VP1 of the same norovirus strain, GII.4/MD145. This antibody recognized the Ruc-MD145 fusion protein by IFA, consistent with the authentic presentation of this conformational epitope in the fusion protein (Fig. 1D). As expected, Ruc-norovirus antigen fusion proteins were not detected in mock-transfected cells (Fig. 1, C and D).

3.2 LIPS and ELISA detection of HuNoV-specific serum IgG antibody titers

The LIPS assay is based on the quantification of virus-specific serum antibodies by measuring the luminescence emitted from *Renilla* luciferase fused to an antigen of interest. Checkerboard titrations of Ruc-antigen concentration (input RLUs) against serially diluted homotypic hyperimmune guinea pig serum showed that an input RLU of 10^7 emitted from the Ruc-antigen was optimal for serum antibody detection (data not shown). To assess the

ability of the LIPS assay to detect HuNoV-specific antibodies in a quantitative format, antibody titers of guinea pig hyperimmune sera raised against GII.4/2006b (Fig. 2A) or GII.4/2004 (Fig. 2C) VLPs were analyzed using GII.4/2006b Ruc-VP1 as antigen. Comparatively, the ELISA was optimized by titrating GII.4/2006b VLP concentration (ng) against the same dilutions of serum (Fig. 2, B and D), and 100 ng was determined as optimal (data not shown). The baseline for the LIPS or ELISA, respectively, was determined by the average RLUs or OD values of unimmunized pooled sera, and the cutoff was determined as the average RLUs or OD values of unimmunized pooled sera plus three standard deviations. Using the LIPS assay, antibody titers for both hyperimmune sera reached the baseline at a serum dilution more than one $\log_{10}$ unit higher than that measured by the ELISA (Fig. 2). In the anti-GII.4/2006b serum, the LIPS measured antibody titers that reached the cutoff value at a serum dilution of 6.55×10^5 , while the ELISA measured titers that reached the cutoff value at a dilution of 1.64×10^5 (Fig. 2, A and B). In the anti-GII.4/2004 serum, both the LIPS and the ELISA measured titers that reached the cutoff value at a serum dilution of 6.55×10^5 (Fig. 2, C and D).

3.3 HuNoV-specific antibody responses following immunization with a multivalent VLP vaccine formulation

To assess whether the LIPS is useful for profiling the adaptive immune response to vaccines, serum antibodies recognizing the GII.4/2006b Ruc-VP1, Ruc-S, and Ruc-P antigens were measured in two minipigs (1584 and 2718) that were immunized with a multivalent formulation of VLPs (Table 1). Three booster doses were given at post-immunization weeks (PIW) 2, 4, and 9. Sera were collected before immunization and showed low HuNoV-specific antibody titers (10 to 40) by VP1-specific ELISA. Additional sera samples were also collected at five time points post-immunization until the study was terminated (PIW 2, 4, 7, 11, and 15). Using a cutoff value calculated from unimmunized sera and the respective antigen, as described in Section 2.5.1, the MFR of HuNoV-specific antibody increased over time against Ruc-VP1, Ruc-S, and Ruc-P, but not against Ruc alone, in both minipigs (Fig. 3). Minipig 1584 (Fig. 3A) developed an earlier and overall higher serum antibody response than minipig 2718 (Fig. 3B) against the GII.4/2006b Ruc-antigens. Antibody responses continued until termination, PIW 15, likely due to a booster dose at a late time-point (PIW 9). These results indicate that this set of Ruc-antigens could track the development of a serologic response to the multivalent formulation, and that antibodies recognizing individual capsid domain proteins could be measured using the LIPS assay.

The specificity of the antibody response to each component of the multivalent formulation was examined by the LIPS assay with Ruc-VP1 constructs representing each component (Tables 1 and 2, Fig. 1B). Each Ruc-VP1 LIPS assay was used to analyze the reactivity of GII.4/2004 hyperimmune serum raised in guinea pigs (Fig. 4A) or pooled minipig multicomponent immunization sera collected at PIW 15 (Fig. 4B). Each individual LIPS assay detected a serologic response in both the GII.4-immunized guinea pig and in the PIW 15-immunized minipigs against each Ruc-VP1 antigen. These data suggested that both homotypic and heterotypic responses were likely detected in the LIPS assay, consistent with the presentation of both specific and shared epitopes among norovirus strains.

To address the relationship between the specific and cross-reactive epitopes detected in the LIPS, we developed a competition format for the assay (Fig. 5A). Homotypic or heterotypic VLPs were incubated with the multicomponent immunization sera prior to the addition of Ruc-VP1 antigen. Antibodies binding to the VLPs would be unavailable, or blocked, for binding to the Ruc-VP1 antigens. Multiple VLP concentrations were tested for their ability to block Ruc-VP1 luminescence, using GI.1 VLP and GI.1 Ruc-VP1 as representative samples (Fig. 5B). A plateau in decreasing RLUs was reached at 1 µg of VLPs, and RLUs did not exhibit any further decrease at VLP concentrations greater than 2 µg. Accordingly, a VLP concentration of 2 µg was used for further competitive assay testing.

The multicomponent immunization sera at PIW 15 were tested by the competitive LIPS using the panel of different Ruc-VP1 antigens. For a preliminary assessment of assay specificity, a competition assay using homologous VLPs was performed (Fig. 6A). Using the cutoff value, which was calculated from mock immunization minipig sera as described in Section 2.5.1, the LIPS assay was able to detect antibodies against GI.1, GI.5, and GII.6 Ruc-VP1 antigens. The percent inhibition of serum antibody binding to the Ruc-VP1 antigen was greater than 95% for each corresponding homologous VLP used (Fig. 6B), except for the two GI VLPs (GI.1 or GI.5; 94% inhibition) and one GII VLP (GII.6; 83% inhibition).

A heterologous VLP competition assay using the GI.1 Ruc-VP1 antigen was also performed. Different combinations of GI VLPs (GI.1, GI.5, and/or GI.6) were used for these tests to compete for antibody binding (Fig. 6C). As expected, the LIPS assay produced a significantly higher detection of serum antibodies against the GI.1 Ruc-VP1 antigen when competing against heterologous VLPs (GI.5 and/or GI.6), compared to homologous VLP competition (Fig. 6C). Interestingly, any combination of homologous with heterologous VLPs also significantly blocked serum antibody binding. The percent inhibition was greater than 95% when the homologous and heterologous VLPs competed for serum antibody binding (Fig. 6D), while the percent inhibition by heterologous VLPs, alone or in combination, was lower than 50%.

4. DISCUSSION

In this study, a LIPS assay was established for the detection of HuNoV-specific serum antibodies against the major capsid protein of multiple HuNoV strains. Preliminary assessment of the HuNoV LIPS showed that the assay had sensitivity similar to the ELISA and could be implemented to perform similar diagnostics or detection of HuNoV-specific antibody levels in serum.

The LIPS assay demonstrated that it could track post-immunization serum antibody responses over time, and detect differences in the levels of serum antibodies recognizing the domains within the HuNoV major capsid (Fig. 3). The Ruc-antigens used in these assays were of the GII.4/2006b strain, which was a HuNoV variant not used in the multicomponent immunization of the minipigs. Interestingly, the levels of serum antibodies recognizing GII.4/2006b Ruc-VP1 and Ruc-S appeared to correlate for both minipigs and across each time point tested. Antibody levels increased until terminal PIW 15 for those directed against Ruc-VP1 and Ruc-S, but not Ruc-P. These results suggest that the Ruc-VP1 and Ruc-S antigens

were similar in their ability to detect antibodies directed against conserved epitopes, compared to Ruc-P which would contain more variable epitopes.

Although the minipigs were immunized with different doses of multiple HuNoV strains, the LIPS assay demonstrated comparable measurements of serum antibodies recognizing each individual strain. These data implicate that the LIPS assay may have detected the presence of antibodies recognizing epitopes on the VP1 that are shared among diverse norovirus strains. The LIPS assay developed here should allow sensitive, high throughput screening of HuNoV antibodies developed following exposure to the virus or vaccines. As understanding of the antigenic topology of noroviruses increases, the LIPS antigens may be further refined to allow the detection of genotype-specific, cross-reactive, or protective antibodies. A high-throughput, sensitive, serological assay such as the LIPS would be able to rapidly screen a large number of sera, such as from a serum bank, to identify samples to be used for further study. Additionally, screening HuNoV-specific antibodies across multiple different infection sera would be useful for understanding how the overall antibody response to HuNoV antigens affects protective immunity.

To understand the affinity of HuNoV-specific antibodies for the Ruc-VP1 antigen, compared to VLPs, which are commonly used in the solid-phase ELISA, a competitive format of the LIPS assay was developed. First, competition was optimized for the VLP concentration that produces the maximum inhibition of serum antibody binding. Then, VLPs that were homologous to different Ruc-VP1 HuNoV strains were used to screen antibodies recognizing the panel of constructs. Interestingly, even after incubation with the homologous VLP, the LIPS assay was able to detect serum antibodies that recognized the GI.1, GI.5, and GII.6 Ruc-VP1 antigens. Accordingly, these data indicate that serum antibodies may recognize certain Ruc-VP1 antigens similarly or even with a better avidity than VLPs in liquid phase, given that intact norovirus VLPs bury certain conformational epitopes (Hansman et al. 2012) that could otherwise be exposed on a single VP1 monomer.

Further evaluation of LIPS assay specificity in detecting strain-specific serum antibodies required inhibition experiments using homologous and heterologous VLPs in competition. Detection of serum antibodies was significantly lower when the homologous GI.1 VLP was used in combination with other GI VLPs, compared to when the heterologous VLPs were used alone or in combination (Fig. 6C and D). The absence of an additive effect in competition assays with GI.5 and GI.6 VLPs could be explained by the existence of cross-reactive epitopes on the GI.5 and GI.6 VP1s and by the saturating amount of VLPs used for the inhibition experiments. Of interest, antibodies recognizing epitopes shared only by the GI.5 and GI.6 VLPs would not have affected the detection of strain-specific antibodies directed against the GI.1 VP1 on the Ruc-antigen.

A limitation of our current study included the possibility of protein misfolding of the HuNoV antigen due to interactions with the Ruc protein, which could affect binding to serum antibodies. However, mAbs specific to a conformational epitope on the P-domain was used by IFA to verify proper expression in the GII.4/MD145 Ruc-VP1. Of interest, the limit of detection for this mAb in the LIPS assay was found to be 1–4 ng (data not shown). It should be noted that the VP1 in the self-assembled VLPs used as coating antigen for ELISA

analyses contained an engineered C-terminal fused His-tag that may have affected how the HuNoV-specific antibodies recognized VLP epitopes in the ELISA. It is also important to note that two luminometers were used in this study. For one luminometer (Synergy), lower RLUs were measured, but when standardizing read time for the data collected, RLUs were similar between both luminometers (data not shown). Overall, the LIPS assay was able to reproduce measurements of trends of HuNoV-specific serum antibody responses across various luminometers and different settings, which should prove useful for utilizing and standardizing the LIPS across different laboratories.

In summary, a LIPS assay was established for the detection of HuNoV-specific antibodies against the VP1 major capsid, and its different domains, of multiple HuNoV variants. The LIPS assay exhibited sensitivity similar to or even superior than the ELISA when measuring serum antibody titers. Using a panel of Ruc-antigen of different HuNoV strains, the LIPS assay and its competitive format showed that the assay can detect strain-specific and/or cross-reactive antibodies. The HuNoV LIPS assay is a high-throughput and sensitive method that should prove useful for profiling serum antibodies recognizing diverse HuNoV strains. Thus, further research is warranted for its refinement, such as to detect antibodies in stool or IgA antibodies (Burbelo et al. 2012). Such information would facilitate future studies elucidating the immunogenic role of different regions of the major capsid protein, as well as the understudied host antibody responses against HuNoV nonstructural proteins to advance vaccine and antiviral development.

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Highlights

- A luciferase immunoprecipitation system (LIPS) assay was developed for the detection of antibodies against human norovirus (HuNoV).
 - The LIPS assay detected higher end-point titers in serum than an ELISA.
 - The LIPS assay allowed comparative analyses of the immune response to individual capsid protein domains.
 - A competitive LIPS assay proved useful in further assessment of the antibody specificity in complex polyclonal sera.

Figure 1. Expression of Ruc-antigen fusion proteins by COS1 cells confirmed by (A, B) western blot and (C, D) immunofluorescence

(A) Western blot of anti-Renilla antibody stained against untransfected, mock lysates and lysates of cells expressing Ruc, or Ruc-VP1, Ruc-S, or Ruc-P of a representative HuNoV strain (GII.4/2006b). (B) Western blot of anti-Renilla antibody stained against a panel of Ruc-VP1 antigen derived from multiple HuNoV strains. (C) Immunofluorescence stains of cells transfected with GII.4/2006b pRuc constructs. Untransfected cells were used as the negative control. Anti-Renilla antibody or hyperimmune guinea pig serum raised against the GII.4/2006b was used to detect fusion protein expression. (D) Immunofluorescence stain of cells transfected with GII.4/MD145 pRuc-VP1. pRuc was used as the negative control. A monoclonal antibody specific to a conformational epitope in the P domain was used to detect protein expression.

Figure 2. HuNoV-specific serum IgG antibody titers of (A, B) anti-GIL.4/2006b or (C, D) anti-GIL.4/MD2004 hyperimmune guinea pig serum measured by the (A, C) LIPS or (B, D) ELISA, using 10^7 RLU of GIL.4/2006b Ruc-VP1 or 100 ng VLPs

Sera were diluted four-fold. Measurements were represented as the geometric mean RLUs (LIPS) or OD (ELISA) for two or more independent runs. Pooled, unimmunized guinea pig sera and the respective antigen were used as the negative control. The solid, horizontal black line represents the baseline, and was calculated as the average RLUs or OD of the negative control data. The dashed, horizontal black line represents the cutoff value, which was calculated as the average RLUs or OD of the negative control data plus three standard deviations. Error bars represent geometric standard deviation for multiple independent runs ($n = 2$). Error bars are not shown for data points if shorter than the height of the symbol. The Synergy luminometer was used for these LIPS data.

Figure 3. LIPS analysis of HuNoV-specific serum IgG antibody responses against GII.4/2006b Ruc-VP1, Ruc-S, and Ruc-P antigens of immunized minipigs identified as (A) 1584, or (B) 2718 HuNoV-specific antibody detection was presented as the geometric mean fold rise of RLUs of two or more independent LIPS assay runs for each minipig, and plotted on a logarithmic scale. Background was determined as the RLU of protein A/G beads, negative control pre-immunization sera, and the respective Ruc-antigen. The fold rise was calculated by dividing the RLU value of tested serum samples by the background. The black, horizontal dashed line represents the cutoff value for positive HuNoV-specific antibody detection. The cutoff value was calculated as the geometric mean fold rise of serum samples ($n = 4$) of a mock immunized pig plus 3 standard deviations. Error bars represent the geometric standard deviation of the mean fold rise for two or more independent runs. Error bars are not shown for data points if shorter than the height of the symbol. The black arrows represent the PIW at which booster doses were given. The Berthold luminometer was used for these LIPS data.

Figure 4. Preliminary assessment of LIPS assay specificity in (A) anti-GII.4/2004 hyperimmune, or (B) multicomponent immunization sera

HuNoV-specific serum IgG antibodies against Ruc-VP1 of 9 different HuNoV strains were measured in hyper- (guinea pig) and multicomponent (minipig) immunization sera. HuNoV-specific antibody detection was presented as the geometric mean fold rise of RLUs of two or more independent LIPS assay runs, and plotted on a logarithmic scale. Background was determined as the RLUs of protein A/G beads, negative control pre-immunization sera, and each Ruc-VP1 antigen. The fold rise was calculated by dividing the RLU value of tested serum samples by the background. The cutoff value was calculated as the geometric mean fold rise of mock immunized minipig serum samples from multiple independent runs ($n = 2$ to 16) plus three standard deviations. Error bars represent geometric standard deviation of the mean fold rise for two or more independent runs. The black, horizontal dashed line represents the cutoff value for positive HuNoV-specific antibody detection. The Synergy luminometer was used for these LIPS data.

Figure 5. Competitive LIPS assay setup

(A) For the competitive LIPS assay, VLPs were added to the serum mixture (40 μ L of buffer A, 10 μ L of the 1:10 diluted serum) overnight at 4 degrees prior to the addition of Ruc-antigen. 10^7 RLU's of Ruc-antigen was then added and incubated for 1 hr at RT. The mixture was transferred to filter plates pre-coated with protein A/G beads. Protein beads precipitate the serum antibodies, and subsequent washes filter the unbound VLP and unbound Ruc-antigen through the wells. Coelenterazine substrate is added, and only antibody complexes with Ruc-antigen luminesce. (B) Different concentrations of GI.1 VLPs were titrated against serum samples from two immunized minipigs at terminal bleed. The detection of HuNoV-specific antibodies was measured using the GI.1 Ruc-VP1 LIPS assay. HuNoV-specific antibody detection was presented as the geometric mean of RLU's of serum samples ($n = 2$) of the two immunized minipigs for more than 4 independent LIPS assay runs, and plotted on a logarithmic scale. Error bars represent the geometric standard deviation of mean RLU's for multiple independent runs ($n = 4$ to 16). The Synergy luminometer was used for these LIPS data.

Figure 6. Competitive LIPS assay (A, C) and percent inhibition analyses (B, D) of HuNoV-specific serum IgG antibodies against (A) Ruc-VP1 of 9 different HuNoV strains and the homologous VLP, or (C) GL1 Ruc-VP1 antigen and different combinations of GI VLPs HuNoV-specific antibody detection was presented as the arithmetic mean of RLUs of more than four independent LIPS assay runs for each minipig at terminal bleed PIW 15, and plotted on a logarithmic scale. Background was determined as the RLU of protein A/G beads, pre-immunization minipig serum samples, and each Ruc-VP1 antigen and VLP. Average RLUs were calculated by subtracting the background RLUs from RLUs of the tested sample. The cutoff value was calculated as the average RLUs of mock immunized minipig serum samples incubated with each Ruc-VP1 antigen and VLP, plus three standard deviations. The black, horizontal dashed line represents the cutoff value for positive HuNoV-specific antibody detection, and error bars represent standard error of the arithmetic mean RLUs ($n = 4$ to 16 independent runs). An asterisk represents statistically significant differences ($p < 0.05$) in mean RLUs among groups by Tukey's multiple comparisons test in one-way ANOVA. The Synergy luminometer was used for these LIPS data.

Table 1

Composition of multivalent HuNoV VLP vaccine for minipig immunization

HuNoV strain	Dose 1 (µg) PIW 0	Dose 2 (µg) PIW 2	Dose 3 (µg) PIW 4	Dose 4 (µg) PIW 9	GenBank Accession Number
Hu/NoV/GI.1/Norwalk/1968/US	75	75	75	75	M87661
Hu/NoV/GI.5/SzUG1/1997-99/JP	44	44	44	44	AB039774
Hu/NoV/GI.6/HSS3/1997/DE	34	34	34	34	AF093797
Hu/NoV/GII.1/Hawaii/1971/US	75	75	75	75	U07611
Hu/NoV/GII.2/SnowMountain/1976/US	75	75	75	75	U70059
Hu/NoV/GII.3/CHDC2005/1975/US	75	75	75	75	HM072045
Hu/NoV/GII.4/MD145-12/1987/US	75	75	75	75	AY032605
Hu/NoV/GII.6/BethesdaD1/2012/US	75	75	75	75	KY424341
Hu/NoV/GIV.1/SaintCloud624/1998/US	20	20	20	20	AF414427
Total concentration of dose (µg):	548	548	548	548	

Table 2

Oligonucleotide sequences used for pRuc-antigen constructs

HuNoV strain	VP1 forward primer	VP1 reverse primer	P-domain forward primer	S-domain reverse primer
Hu/GIL.4/2006b/092895/2008/US	5'-TTAAATGGATCC ¹ ATGAAGATGGCGTCGAATGAC-3' (nt 1-21) ³	5'-ATTAAAGCGGCCGC- <u>CTA</u> ² TTATAATGCACGCTCTACGCCCGC-3' (nt 1601-1623) ³	5'-TTAAATGGATCC ¹ TTTTTGGTGCCGCCTACAGT 3' (nt 640-659) ³	5'-ATTAAAGCGGCCGC- <u>CTA</u> ² TTATATGAAGTCAAAAATCAGGGG-3' (nt 620-640) ³
Hu/NoV/GIL.1/Norwalk/1968/US	5' ATCTAAAAAGGTT ¹ GCGTCTAAAGGACGCTACATCAAGC 3' (nt 10-33) ³	5' TATGTACTCGAG- <u>TCA</u> ² TCGGCGCAGACCAAGC 3' (nt 1575-1590) ³		
Hu/NoV/GIL.5/SzUGI/1997-99/JP	5' TTAAATGGATCC ¹ ATGATGATGGCGTCTAAAGGA 3' (nt 1-20) ³	5' ATTAAAGCGGCCGC- <u>CTA</u> ² TTATCTCTCTGATGCCAAGCC 3' (nt 1613-1632) ³		
Hu/NoV/GIL.6/HSS3/1997/DE	5' TTAAATTTCTAGA ¹ ATGATGATGGCGTCTAAAGGA 3' (nt 1-20) ³	5' ATTAAAGCGGCCGC- <u>CTA</u> ² TTATATGCGCCCGAAGACCAA 3' (nt 1604-1623) ³		
Hu/NoV/GIL.1/Hawaii/1971/US	5' TTAAATGGATCC ¹ ATGAAGATGGCGTCGAATGA 3' (nt 1-20) ³	5' ATTAAAGCGGCCGC- <u>CTA</u> ² TTACTGGACTCTTCTGCG 3' (nt 1591-1608) ³		
Hu/NoV/GIL.2/SnowMountain/1976/US	5' TTAAATGGATCC ¹ ATGAAGATGGCGTCGAATGA 3' (nt 1-20) ³	5' ATTAAAGCGGCCGC- <u>CTA</u> ² TTACTGAACCTTCTACGCC 3' (nt 1610-1629) ³		
Hu/GIL.3/CHDC2005/1975/US	5' TTAAATGGATCC ¹ ATGAAGATGGCGTCGAATGA 3' (nt 1-20) ³	5' ATTAAAGCGGCCGC- <u>CTA</u> ² TTATGAATCCTTCTACGCC 3' (nt 1627-1647) ³		
Hu/NoV/GIL.4/MD145-12/1987/US	5' ATCTAAAAAGGTT ¹ GCGTCGAGTGACGCCAAC 3' (nt 10-27) ³	5' TATGTACTCGAG- <u>TCA</u> ² TAATGCACGCTGCGCC 3' (nt 1601-1620) ³		
Hu/GIL.6/BethesdaD1/2012/US	5' TTAAATGGATCC ¹ ATGAAGATGGCGTCGAATGA 3' (nt 1-20) ³	5' ATTAAAGCGGCCGC- <u>CTA</u> ² TTATGAACCTTCTTTCGCCCC 3' (nt 1623-1644) ³		
Hu/NoV/GIV.1/SaintCloud624/1998/US	5' TTAAATCTCGAG ¹ ATGAAGATGGCGTCGAGTGA 3' (nt 1-20) ³	5' ATTAAAGCGGCCGC- <u>CTA</u> ² TTATGAAACCTCACTTACCCCTT 3' (nt 1647-1671) ³		

¹Restriction sites used for subcloning are underlined.²Sequences corresponding to stop codons are shown in bold.

The nucleotide numbers given in parentheses correspond to ORF2 numbering systems.

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