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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.

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 et al., 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, 2008).

Assessing neutralizing antibodies and their role in protection and immunity has been difficult in the absence of fully permissive cell

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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 et al., 2015; Hutson et al., 2002, 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.4 2006b/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

Table 1
Composition of multivalent HuNoV VLP vaccine for minipig immunization.

HuNoV strain	Dose 1 (μg)	Dose 2 (μg)	Dose 3 (μg)	Dose 4 (μg)	GenBank Accession Number
	PIW 0	PIW 2	PIW 4	PIW 9	
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	

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