Serological Responses to a Norovirus Nonstructural Fusion Protein after Vaccination and Infection

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Running Title: NoV VPR Antibody Responses

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The performance of an assay to detect antibodies to a norovirus nonstructural fusion protein, designated VPR and consisting of three proteins (GI.1 virus protein-genomic [VPg], virus protease and a RNA-dependent, RNA polymerase), was evaluated. Assay sensitivity and specificity were 74.5% and >95%, respectively, in identifying GI.1 norovirus infection among persons who received either a monovalent GI.1 norovirus VLP vaccine or placebo by the intranasal route followed by oral live GI.1 norovirus challenge.
Noroviruses cause an estimated 20 million infections per year in the United States and are the leading cause of non-bacterial gastroenteritis worldwide (1,2). The norovirus genome consists of 3 open reading frames (ORFs). ORF1 codes for several non-structural proteins that are transcribed as a polyprotein before being cleaved into individual proteins by viral protease. ORF2 and ORF3 code for structural capsid proteins VP1 (the major capsid protein) and VP2 (a minor capsid protein), respectively (3).

A candidate vaccine has been developed that is based upon immunization with a norovirus VP1 protein (4). Norovirus infection can be detected by measuring seroresponses to the VP1 protein (5), but the sensitivity of serological detection targeting the VP1 protein and the diagnostic precision of this test is diminished by prior immunization (6). We previously developed a serological assay targeting a nonstructural protein, the norovirus protease, and the sensitivity of the assay was 53% when using a homologous protease as antigen (7). Preliminary studies indicated that although the viral polymerase induced seroresponses less frequently than the viral protease protein, a fusion protein (VPR) consisting of three non-structural viral proteins (VPg plus protease plus RNA-dependent, RNA polymerase) was able to detect seroresponses at a higher frequency than we reported for the viral protease alone. In the current study, we evaluated the performance of a serological assay using the VPR fusion protein to identify infection among persons who participated in a GI.1 norovirus candidate vaccine trial (LV01-103) that included a live oral challenge with a GI.1 norovirus (8).

Serum samples were included in the current study from persons in the LV01-103 study who gave permission for future use of collected samples and participated in the challenge portion of the study. Serum samples from pre-vaccination (day 0), post-vaccination 1 (day 21), post-vaccination 2 and pre-challenge (day 42 or later), and post-challenge (day 30 post-challenge) were tested by ELISA for the presence of antibody to the fusion protein, VPR. Full length VPR was PCR-amplified from the Norwalk virus genome (Genbank NC_001959) and cloned into pET46 Ek/LIC vector (EMD Millipore). Mutations E138A, C277A and E319A were introduced with a site-directed mutagenesis kit (Stratagene) to
completely inactivate the viral protease (C277A) and prevent auto proteolysis by removing the cleavage sites (E138A, E319A). The protein was then expressed in the BL21 DE3 strain of *E. coli*. The integrity and purity of the polyprotein product was confirmed using a Coomassie-stained, SDS-PAGE gel which demonstrated a single ~100 kilodalton (kD) product, the expected size of the VPg (20 kD), protease (18 kD) and RNA polymerase (56 kD) fusion protein. Purification was achieved with a combination of Ni-affinity, SP sepharose ion exchange, and S200 gel filtration chromatography into a final buffer containing 20mM Tris (pH: 8.0), 300mM NaCl, 5mM MgCl₂ and 1mM DTT. Endpoint dilutions were performed to determine antibody titers, and the relationship between serial titers in individual patients was evaluated. In brief, 100 ng of VPR protein diluted in 0.01 M phosphate buffered saline (PBS) was coated onto 96-well polyvinyl chloride plates (Thermo Scientific) for 4 hours at room temperature, and the plates were blocked overnight at 4°C with 10% blotto in 0.01 M PBS. After washing with 0.01 M PBS plus 0.05% Tween-20 (PBS-Tween), sera diluted from 1:20 to 1:1280 in PBS-5% blotto were added to duplicate wells and incubated for 1 hour at 37°C. After washing, anti-VPR antibodies were detected with a 1:5000 dilution of peroxidase-labeled goat anti-human antibody (IgA, IgG, IgM) (KPL) in PBS-5% blotto. The reaction was developed by addition of a mixture of TMB Peroxidase Solution (KPL) and stopped after 10 min by addition of 1M H₃PO₄. The optical density was determined with a spectrophotometer at a wavelength of 450 nm, and the cutoff for a positive sample was an OD greater than 0.1. A positive control serum from a Norwalk virus-infected person infected in a separate study (S) was run on each plate, and all samples from the individual were run in the same experiment.

Sera were available for study from 73 of the 84 persons who participated in the challenge study; 67 were in the per protocol analysis, which is reported here since the intent-to-treat (ITT) and per protocol analyses yielded similar results. All subjects had anti-VPR antibody detected at the time of study enrollment. Seroresponse frequency (four-fold changes in antibody level) was assessed following vaccination and following challenge regardless of whether infection occurred. The gold standard for
infection was the definition used in the LV01-103 study (8); all persons in the current analysis had fecal shedding of Norwalk virus as detected by RT-PCR and a four-fold or greater increase in total serum ELISA antibody titer to Norwalk virus. Thirty-eight of 51 infected persons had a four-fold or greater increase in serum antibody to the VPR protein, with a geometric mean fold rise of 5.3 (Table 1). Anti-VPR seroresponse frequencies were similar among infected persons who did or did not meet the protocol-defined definition of gastroenteritis (76.3% and 69.2%, respectively). In contrast, none of the 16 persons who were not shown to be infected after challenge had a seroresponse to the VPR protein. Sensitivity and specificity of the VPR antibody assay following Norwalk virus challenge were 74.5% and 100%, respectively.

We also looked at seroresponses after vaccination. Because the vaccine only contained the Norwalk virus VP1 protein, seroresponses to the VPR protein are not expected to occur. No seroresponses to the VPR protein were observed after the first inoculation of study product, while three occurred after the second (two in vaccine recipients and one in a placebo recipient) (Figure 1). Thus, the specificity of the assay following vaccination for the total study population was 95.5%.

Each of the three persons who had a seroresponse to the VPR protein also had a seroresponse to the VP1 protein, raising the possibility that these subjects had subclinical infection with another norovirus strain during the interval. Assay variability is another possibility; the three subjects were in both treatment groups. We have shown previously that serological responses to the viral protease are cross-reactive between genogroups (7), and a single study participant in the ITT group, a placebo recipient who was symptomatically infected with a GII.4 norovirus, but not the GI.1 challenge strain, during the challenge portion of the study also seroresponded to the VPR protein. This latter subject developed gastroenteritis symptoms 19 minutes after receipt of the live GI.1 virus by oral challenge (8).
In summary, 74.5% of persons infected with a GI.1 norovirus had a seroresponse to the norovirus nonstructural fusion protein, VPR, while the diagnostic specificity of the assay was greater than 95%. The sensitivity of the norovirus VPR antibody assay was higher than the 53% reported previously using the Norwalk viral protease alone as antigen (7), and it has potential utility in differentiating serological responses to inactivated and subunit norovirus vaccines from those induced by wild type norovirus infection.

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Figure Legend

Figure 1. Seroresponse frequencies to capsid and VPR fusion proteins by vaccination group.

Seroresponse frequencies to the capsid (VP1) antigen and to the fusion protein consisting of VPG, viral protease and the viral RNA-dependent RNA polymerase (VPR) are shown among norovirus vaccine (Vacc) and placebo (Pla) recipients. Time intervals include before and after the first vaccination (Day 0-21, white bar), before and after the second vaccination (Day 21-42, stippled bar), and before the first vaccination to after the second vaccination (Day 0-42, solid bar). The bars represent 95% confidence intervals around the observed frequencies.


<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Pre-challenge GMT (95% CI)</th>
<th>Post-challenge GMT (day 30) (95% CI)</th>
<th>GMFR from Pre- to Post-challenge (95% CI)</th>
<th>Post-challenge Seroresponse Frequency* (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infected</td>
<td>51</td>
<td>84 (65, 110)</td>
<td>449 (339, 596)</td>
<td>5.3 (4.1, 7.0)</td>
<td>74.5% (60.4%, 85.7%)</td>
</tr>
<tr>
<td>Gastroenteritis</td>
<td>38</td>
<td>79 (59, 105)</td>
<td>461 (337, 630)</td>
<td>5.9 (4.3, 8.0)</td>
<td>76.3% (59.8%, 88.6%)</td>
</tr>
<tr>
<td>No gastroenteritis</td>
<td>13</td>
<td>104 (53, 205)</td>
<td>418 (204, 855)</td>
<td>4.0 (2.2, 7.2)</td>
<td>69.2% (38.6%, 90.9%)</td>
</tr>
<tr>
<td>Not infected</td>
<td>16</td>
<td>135 (64, 283)</td>
<td>129 (63, 262)</td>
<td>1.0 (0.8, 1.1)</td>
<td>0% (0%, 20.6%)</td>
</tr>
<tr>
<td>P value (Infected vs. not infected)</td>
<td>0.134 &lt;0.001 &lt;0.001 &lt;0.001</td>
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*Four-fold or greater antibody titer rise to VPR protein from pre-challenge
AUTHOR CORRECTION

Correction for Hesse et al., Serological Responses to a Norovirus Nonstructural Fusion Protein after Vaccination and Infection

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