Relationship between genotypes and serotypes of genogroup 1 recoviruses: a model for human norovirus antigenic diversity

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Human norovirus (NoV) research greatly relies on cell culture-propagable surrogate caliciviruses, including murine NoVs and the prototype ‘recovirus’ (ReCV), Tulane virus. However, the extreme biological diversity of human NoVs cannot be modelled by a uniform group of viruses or single isolate. Based on a diverse group of recently described ReCVs, a more advanced model reflecting human NoV biological diversity is currently under development. Here, we have reported the genotypic and serotypic relationships among 10 G1 ReCV isolates, including Tulane virus and nine other recent cell culture-adapted strains. Based on the amino acid sequences of virus capsid protein, VP1, and classification constraints established for NoVs, G1 ReCVs were separated into three genotypes, with variable organization of the three open reading frames. Interestingly, cross-neutralization plaque assays revealed the existence of four distinct serotypes, two of which were detected among the G1.2 strains. The amino acid (aa) difference between the two G1.2 ReCV serotypes (12%) was less than the minimum 13% difference established between NoV genotypes. Interestingly, one of the G1.3 ReCVs was equally neutralized by antisera raised against the G1.3 (6% aa difference) and G1.1 (25% aa difference) representative strains. These results imply the existence of a large number of human NoV serotypes, but also shared cross-neutralization epitopes between some strains of different genotypes. In conclusion, the newly developed ReCV surrogate model can be applied to address biologically relevant questions pertaining to enteric CV diversity.

INTRODUCTION

Caliciviruses (CV) are non-enveloped, positive-sense, single-stranded RNA viruses with polyadenylated genomes of ~6.5–8.5 kb. The Caliciviridae family consists of five established genera: Norovirus, Sapovirus, Lagovirus, Vesivirus and Nebovirus. Three recently proposed CV genera, ‘Recovirus’, ‘Valovirus’ and ‘chicken calicivirus’, are awaiting ICTV approval (Green, 2013). Depending on the genus, CV genomes are organized into two or more ORFs. Noroviruses (NoVs) and vesiviruses encode nonstructural (NS) and capsid (VP1) proteins in separate ORFs (ORF1 and ORF2, respectively), while sapoviruses and lagoviruses encode both NS and capsid proteins in a continuous ORF1. A separate ORF encoding a minor structural protein (VP2), designated ORF3 in the noro- and vesivirus genomes and ORF2 in the sapo- and lagovirus genomes, is present in all CVs (Green, 2013). In murine NoV genomes, an additional ORF (ORF4) overlapping ORF2 encodes a protein (VF1) that may play a role in virulence (McFadden et al., 2011).

CVs cause a variety of diseases in animals. In humans, NoVs and sapoviruses, commonly referred to as ‘human CVs’, are two of the leading causes of acute gastroenteritis. In the United States alone, NoVs cause an estimated 23 million cases of acute gastroenteritis, leading to 70,000 hospitalizations and 800 deaths each year (Lopman et al., 2011; Scallan et al., 2011; Hall et al., 2012).

The prototype NoV (Norwalk virus) was discovered more than 40 years ago (Kapikian et al., 1972). However, due to the lack of an efficient cell culture system and robust animal model, human NoV research still relies on surrogate CVs and/or hosts (e.g. murine NoV or gnotobiotic pig/calf) that do not necessarily reflect the essential biological features and diversity of human NoVs and their hosts (Table 1). The chimpanzee model is a promising alternative for the pre-clinical evaluation of NoV vaccines and antivirals (Bok et al., 2011). However, the use of chimpanzees for biomedical research is restricted in most countries worldwide, including...
the USA. At present, licensed vaccines or antiviral strategies to prevent NoV disease are not available. One of the major challenges in NoV vaccine design is the tremendous diversity of human NoVs (Green, 2013). According to phylogenetic differences of the capsid protein, VP1, NoVs are classified into five genogroups (G1–G5), with several genetic types in each genogroup (e.g. G1.1–G1.8). Based on analysis of 164 human and animal NoV VP1 proteins, amino acid (aa) sequence differences established between strains (0–14.07 %) within a genetic type, between genotypes (14.26–43.78 %) and between genogroups (44.91–43.78 %) are used for the genetic classification of new isolates (Zheng et al., 2006; Kroneman et al., 2013). Genogroups G1, 2 and 4 mainly contain human NoVs, while G3 includes bovine and G5 murine NoVs. Over 30 human NoV genetic types have been described to date (Green, 2013). Antigenic characterization of human NoVs mainly relies on virus-like particles (VLP) and antibody binding assays (ELISA). Several studies have demonstrated the existence of cross-reactive conserved epitopes among the different NoV genotypes and even genogroups, predominantly located in the S-domain or C-terminal region of the P1 domain (Parker et al., 2005; Oliver et al., 2006; Parra et al., 2013).

The role of histo-blood group antigens (HBGA) in human NoV binding/attachment and susceptibility to infection, and diversity of the HBGA binding properties of different human NoV strains are well established (Lindesmith et al., 2003; Huang et al., 2005; Frenck et al., 2012). HBGA binding sites have been mapped to the most variable P2 subdomain of the NoV capsid (Choi et al., 2008; Shanker et al., 2011). The surface-exposed P2 subdomain probably also contains the epitopes responsible for major antigenic differences (serotypes). Antibodies that block adherence of human NoV VLPs to HBGAs in binding assays (ELISA) have been proposed as virus-neutralizing (VN) antibodies. Recent results based on blocking assays performed mainly with G2.4 human NoVs suggested that antigenic changes facilitating escape from herd immunity may also drive changes in HBGA binding affinities and lead to altered population susceptibility (Debbink et al., 2012; Lindesmith et al., 2012). However, due to the lack of a human NoV cell culture system, evaluating the roles of HBGAs as attachment ligands or receptors and direct assessment of human NoV antibody neutralization, including the identification of neutralizing epitopes and relationship between genotypes and serotypes, remains a significant challenge.

Murine NoVs are closely related to their human counterparts, replicate well in cell culture and represent a cost-effective small animal model (Wobus et al., 2006). However, the murine NoV model also has limitations highlighted by significant differences compared with human NoVs. Murine NoVs exhibit limited genetic and antigenic diversity, have apparent immune cell tropism and cause persistent asymptomatic infection in normal mice.

The prototype ‘recovirus’ (ReCV), Tulane virus, was discovered in stool samples of juvenile rhesus macaques (Farkas et al., 2008). This initial discovery was followed by the identification of several other ReCVs, including one strain in human stool samples (Farkas et al., 2010a; Handley et al., 2012; Smits et al., 2012). Since then, several additional ReCV strains described in our previous reports (Farkas et al., 2010a) have been cell culture-adapted. Studies on Tulane virus and other isolates established that ReCVs are evolutionarily and biologically closely related to human NoVs, including genetic, antigenic and HBGA binding diversities and clinical symptoms of gastroenteritis observed in experimentally infected macaques (Farkas et al., 2008, 2010a, b; Sestak et al., 2012). Based on the biologically diverse ReCV isolates, we are currently developing a cell culture and animal model that can effectively replicate the biological features of human NoVs. Here, we examined the relationship between

Table 1. Comparison of human NoVs and surrogate caliciviruses, including ReCVs

<table>
<thead>
<tr>
<th>Genus</th>
<th>HuNoV</th>
<th>MuNoV</th>
<th>PoNoV</th>
<th>BoNoV</th>
<th>PECV</th>
<th>FCV</th>
<th>RHDV</th>
<th>ReCV</th>
</tr>
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<tbody>
<tr>
<td>Genome organization</td>
<td>3 ORFs</td>
<td>4 ORFs</td>
<td>3 ORFs</td>
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<td>2 ORFs</td>
<td>3 ORFs</td>
<td>2 ORFs</td>
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</tr>
<tr>
<td>Diversity</td>
<td>High</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>HBGA binding</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Efficient cell culture</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Efficient reverse genetics</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
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<td>Diarrhoea in immunocompetent host</td>
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<td>Yes</td>
<td>Yes</td>
<td>No*</td>
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<td>Shedding in stool</td>
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<td>Yes</td>
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<td>Yes</td>
<td>Yes</td>
<td>No*</td>
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<td>Yes</td>
</tr>
<tr>
<td>Zoonotic transmission</td>
<td>Suggested</td>
<td>No</td>
<td>Suggested</td>
<td>Suggested</td>
<td>No</td>
<td>No*</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Availability from ATCC</td>
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<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

HuNoV: human norovirus; MuNoV: Murine norovirus; PoNoV: porcine norovirus; BoNoV: bovine norovirus; PECV: porcine enteric calicivirus; FCV: feline calicivirus; RHDV: rabbit haemorrhagic disease virus; ReCV: rhesus enteric calicivirus.

*FCV primarily sheds in oral and ocular nasal discharge, but can be found in all body secretions, including faeces, during acute disease.
RESULTS

Phylogeny and genome organization

Phylogenetic analysis of complete ORF2 (VP1) sequences of the three ReCVs available in public databases (Tulane virus, WUHARV and Bangladesh/289/2007) and nine tissue culture-adapted ReCVs obtained in this study confirmed our earlier classification based on short RdRp sequences (Farkas et al., 2010a). Genogroup 1 ReCVs were clearly segregated into three genotypes with evolutionary distances comparable to those between the human NoV genotypes. The amino acid differences between strains of the three genotypes ranged from 24 to 30% (Table 2). In addition to G2 rhesus isolates, the human ReCV isolate, Bangladesh/289, represents a third genogroup (G3) (Fig. 1).

Analysis of the ReCV genomes revealed three ORFs with significant variations in arrangement. When ORF1 was adjusted as frame 1, all G1.1 and G1.2 ReCVs exhibited an order of frames 1, 1 and 3, while the order for G1.3 ReCVs was frames 1, 3 and 2 for ORF1, ORF2 and ORF3, respectively. Human NoVs, murine NoVs and ReCV isolate Bangladesh/289 exhibited an order of frames 1, 2 and 1, with an additional ORF4 in-frame 3 in murine NoV genomes (Fig. 2). The Norwalk virus ORF1 stop and ORF 2 start codons overlapped by 11 nt (ATGATGATGGCGTGTTAA), the murine NoV by 8 nt (ATGAGGATGAGTGA) and ReCV Bangladesh/289 by 146 or 113 nt, depending on which of the two possible start codons was utilized (ATGAGCTCCCAAAAGGAAAATCTGTCAACAAATTGGAGAAGTGGTGCAACGACAACTGGAGGGTTTGAGGATCTAACC-
GGAGCGACCGCCCACACTACGAGAATCCCAAAGGACTCTGAATTGGCCTCCACATCATTGGA). The ORF1 stop and ORF 2 start codons were separated by 21 nt in the G1.1 ReCV genomes (TGATGATCAATT-
GTCATAGACATG), 3 nt in the G1.2 ReCV genomes (TGA-GCTATG) and 2 nt in the G1.3 ReCVs (TGATCATA). ORF2 start and ORF3 stop codons of all NoVs and ReCVs overlapped by 1 nt (TAATG).

G1 ReCV serotypes

Hyperimmune sera against the Tulane virus (G1.1), FT285 (G1.2) and FT7 (G1.3) ReCVs exhibited low-level cross-reactivity. The difference in titres between the homologous and heterologous antisera ranged between 102- and 2560-fold, and was greater for all strains than the 20-fold cutoff used to define serotypes (Table 3). Thus, the three G1 strains used for generating hyperimmune sera represent not only three genotypes but also three serotypes.

Serotypic evaluation of the 10 G1 ReCV isolates was performed in a standardized cross-neutralization plaque assay with 20 virus neutralization units (VNU) of serotype-specific hyperimmune sera. Anti-Tulane virus hyperimmune sera neutralized all G1.1 isolates and FT65, a G1.3 strain, but not the other two G1.3 or the G1.2 strains. Surprisingly, anti-FT285 (G1.2) hyperimmune sera only neutralized FT285, but not the two other G1.2 strains (FT157 and FT499). Anti-FT7 (G1.3) hyperimmune sera neutralized only the G1.3 strains (Fig. 3). Based on these results, the 10 G1 ReCVs were grouped into four serotypes, specifically, serotype 1 (G1.1), serotype 2 (FT285, G1.2), serotype 3 (FT7 and FT400, G1.3) and serotype 4 (FT157 and FT499, G1.2). The exact serotype of FT65 (G1.3) ReCV could not be determined with the antibodies used in this study.

Virus neutralization epitope prediction

Several discontinuous B cell epitopes were predicted in almost all of the 10 ReCV strains in this study. These included PPT in the hinge region (aa positions 206–208 in the FT285 VP1 sequence), AITDKP-RR in loop A’-B’ of the P2 domain (aa positions 283–288) and M-VSG (aa positions 285–288) in the P2 domain.

Table 2. VP1 pairwise amino acid difference and homology values (%) of ReCVs

<table>
<thead>
<tr>
<th></th>
<th>TV</th>
<th>FT205</th>
<th>FT218</th>
<th>FT494</th>
<th>FT285</th>
<th>FT499</th>
<th>WUHARV</th>
<th>FT7</th>
<th>FT65</th>
<th>FT400</th>
<th>Bangladesh</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1.1-TV</td>
<td>–</td>
<td>91</td>
<td>91</td>
<td>91</td>
<td>74</td>
<td>74</td>
<td>75</td>
<td>75</td>
<td>75</td>
<td>75</td>
<td>38</td>
</tr>
<tr>
<td>G1.1-FT205</td>
<td>9</td>
<td>–</td>
<td>97</td>
<td>97</td>
<td>75</td>
<td>75</td>
<td>75</td>
<td>74</td>
<td>74</td>
<td>75</td>
<td>38</td>
</tr>
<tr>
<td>G1.1-FT218</td>
<td>9</td>
<td>3</td>
<td>–</td>
<td>98</td>
<td>75</td>
<td>76</td>
<td>76</td>
<td>76</td>
<td>75</td>
<td>76</td>
<td>39</td>
</tr>
<tr>
<td>G1.1-FT494</td>
<td>9</td>
<td>3</td>
<td>2</td>
<td>–</td>
<td>74</td>
<td>75</td>
<td>75</td>
<td>75</td>
<td>74</td>
<td>75</td>
<td>39</td>
</tr>
<tr>
<td>G1.2-FT285</td>
<td>26</td>
<td>25</td>
<td>25</td>
<td>26</td>
<td>–</td>
<td>88</td>
<td>90</td>
<td>71</td>
<td>70</td>
<td>71</td>
<td>40</td>
</tr>
<tr>
<td>G1.2-FT499</td>
<td>26</td>
<td>25</td>
<td>24</td>
<td>25</td>
<td>12</td>
<td>–</td>
<td>92</td>
<td>71</td>
<td>71</td>
<td>71</td>
<td>40</td>
</tr>
<tr>
<td>G1.2-WUHARV</td>
<td>25</td>
<td>25</td>
<td>24</td>
<td>25</td>
<td>10</td>
<td>8</td>
<td>–</td>
<td>71</td>
<td>71</td>
<td>71</td>
<td>40</td>
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<td>G1.3-FT7</td>
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<td>26</td>
<td>24</td>
<td>25</td>
<td>29</td>
<td>29</td>
<td>29</td>
<td>–</td>
<td>94</td>
<td>97</td>
<td>40</td>
</tr>
<tr>
<td>G1.3-FT65</td>
<td>25</td>
<td>26</td>
<td>25</td>
<td>26</td>
<td>30</td>
<td>29</td>
<td>29</td>
<td>6</td>
<td>–</td>
<td>95</td>
<td>41</td>
</tr>
<tr>
<td>G1.3-FT400</td>
<td>25</td>
<td>25</td>
<td>24</td>
<td>25</td>
<td>29</td>
<td>29</td>
<td>29</td>
<td>3</td>
<td>5</td>
<td>–</td>
<td>39</td>
</tr>
<tr>
<td>G3.1-Bangladesh</td>
<td>62</td>
<td>62</td>
<td>61</td>
<td>61</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>59</td>
<td>61</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Pairwise amino acid difference and homology scores are shown in the lower and upper diagonal half of the table, respectively.
The hinge epitope was predicted in all strains except TV, loop A'-B' epitope in all strains except FT205 and FT494, and M-VSG epitope in all strains except TV, FT7 and FT400. Analysis of multiple sequence alignments highlighted the loop A'-B' epitope (Fig. 4) as the most likely candidate responsible for the VN differences among the strains (Fig. 3).

DISCUSSION

Since the discovery of the prototype Tulane virus (Farkas et al., 2008), a number of similarities have been identified between ReCVs and human NoVs, including close evolutionary relatedness, genetic diversity, HBGA binding properties, epidemiology and disease spectrum (Farkas et al., 2010a, b, 2012; Sestak et al., 2012). Human NoVs cannot be grown in cell culture. For this reason, other CVs are used as surrogates for human NoV research (Vashist et al., 2009; Richards, 2012). Cell culture-propagable murine NoVs have emerged as the most commonly utilized human NoV surrogates (Wobus et al., 2006). However, there remains a need for more robust surrogates, as evident from the ongoing NoroCore projects that form part of the USDA-NIFA Food Virology Initiative (http://norocore.ncsu.edu). These efforts have yielded several recently published studies with the prototype Tulane virus (DiCaprio et al., 2012; Hirneisen & Kniel 2013a, b; Li et al., 2013). One of the major challenges in human NoV research, particularly for vaccine development, is the enormous diversity (genetic, antigenic, HBGA binding) of human NoVs. These features cannot be
duplicated by a uniform group of viruses or single isolate. Based on the availability of a large collection of diverse ReCVs in our laboratory, we recently initiated the development of a cell culture and non-human primate model able to duplicate the diverse biological features of human NoVs and their hosts. In the current study, we evaluated the relationship between the genotypes and serotypes of an enteric CV for the first time using this unique model.

Partial RdRp, whole capsid protein (VP1) and whole minor structural protein (VP2)-encoding sequences were obtained for nine cell culture-adapted G1 ReCVs. Phylogenetic analysis of ORF2 nt and VP1 aa sequences confirmed our previous classification based on short (268 nt) RdRp sequences (Farkas et al., 2010a) and the existence of three genotypes among G1 ReCVs. Amino acid differences among the ReCV genotypes (24–30 %) were within the range (14.26–43.78 %) established for human NoV genotypes (Zheng et al., 2006). ReCV WUHARV was grouped with the G1.3 strains, while the human ReCV isolate, Bangladesh/289, was clearly separated from rhesus isolates with 59–62 % aa differences, equivalent to those reported between the human NoV genogroups (44.91–64.41 %) (Table 2) (Zheng et al., 2006). Genogroup 2 ReCV VP1 sequences have not been published yet. However, based on analyses of published VP1 and/or RdRp sequences, ReCVs

**Table 3. Cross-reactivity of anti-ReCV hyperimmune mouse sera**

<table>
<thead>
<tr>
<th>Reciprocal of VN titres</th>
<th>Anti-TV</th>
<th>Anti-FT285</th>
<th>Anti-FT7</th>
</tr>
</thead>
<tbody>
<tr>
<td>TV</td>
<td>5120</td>
<td>10</td>
<td>40</td>
</tr>
<tr>
<td>FT285</td>
<td>10</td>
<td>25600</td>
<td>40</td>
</tr>
<tr>
<td>FT7</td>
<td>50</td>
<td>&lt;10</td>
<td>12800</td>
</tr>
<tr>
<td>Homologous/</td>
<td>102-fold</td>
<td>2560-fold</td>
<td>320-fold</td>
</tr>
<tr>
<td>heterologous*</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

VN end titres were calculated based on repeated experiments with twofold dilutions, starting from 1:10 and 1:50.

*The least difference of titres of an antiserum against homologous and heterologous strains. Twenty-fold or greater difference in both directions was used to distinguish serotypes.

Fig. 2. Genome organization of ReCVs and Noroviruses. ORFs are shown as boxes. ORF1 encodes non-structural proteins, ORF2 the capsid protein (VP1), and ORF3 the minor structural protein (VP2). In the murine NoV genome, a fourth ORF that overlaps with ORF2 has been identified that encodes virulence factor (VF1) protein (McFadden et al., 2011).
were classified into three genogroups (G1–G3), with three genotypes within G1 (G1.1–G1.3) (Fig. 1). Considering that most known ReCV sequences originated from samples collected from the same colony of captive macaques between April and July of 2008 (Farkas et al., 2010a), the observed genetic diversity indicates highly diverse, human NoV-like evolution of ReCVs. Analysis of the ReCV genomes revealed variable arrangement of the three ORFs among the different ReCV genotypes that was not observed among NoVs (Fig. 2). Further studies using different geographical locations and primate species, including humans, are necessary to determine the full extent of ReCV diversity.

To standardize for cell culture-based serotyping assays, only ReCV strains isolated in the LLC-MK2 cell line were involved in this study. Mouse hyperimmune sera generated against one representative strain of each of the three G1 genotypes were used for serotyping. End titration with the three representative strains used for immunization based on requirements established for picornavirus and rotavirus serotyping (Committee on Enteroviruses, 1962; Kapikian et al., 1967; Wyatt et al., 1982) revealed that these strains represent three different serotypes. The cross-reactivity between Tulane virus (serotype 1) and FT7 (serotype 3) was stronger than between FT285 (serotype 2) or other serotypes. However, the difference in titres in both directions was still at least fourfold higher between all strains than the 20-fold minimum cut-off value established for separating serotypes (Table 3).

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**Fig. 3.** Serotyping of G1 ReCVs. (a) Serotyping plaque assay. Twenty virus neutralization units (VNU) of each hyperimmune mouse serum were used to cross-neutralize 50–100 p.f.u. of ReCV. (b) Mean plaque reduction (%) of repeat experiments. Error bars represent standard deviation. (*) Shows statistically significant difference (P<0.05), compared with neutralization by homologous virus/serum. (●) Indicates neutralization by genotype-matched sera. (◇) Indicates lack of neutralization by genotype-matched sera. (◇) Indicates neutralization by sera raised against a heterologous genotype. Serotypes were established based on ≥80% plaque reduction.
Due to the limited volume of mouse hyperimmune sera, antigenic relationships between the 10 ReCV isolates were evaluated in a standardized cross-neutralization plaque assay. To adjust for differences in VN antibody levels in individual hyperimmune serum samples, the test was performed with a single dilution of each serum (representing 20 VNUs) against the homologous strain (Cowen & Hitchner, 1975; Smith et al., 1998). The cross-neutralization assay highlighted statistically significant differences among the ReCV isolates. All the G1.1 isolates belonged to serotype 1. The anti-FT285 (serotype 2) serum was not able to neutralize the other two G1.2 strains (FT157 and FT499), leading to separation of the G1.2 isolates into two serotypes (serotypes 2 and 4). All G1.3 strains were neutralized by anti-FT7 (serotype 3) serum, and consequently grouped as serotype 3. One of the G1.3 isolates (FT65) was also neutralized by serotype 1-specific serum (Fig. 3). This finding is of significant interest, since FT65 and FT7 differ only by 6 %, while FT65 and Tulane virus differ by 25 % in terms of VP1 aa positions (Table 2). Heterologous sera exhibited significantly lower neutralization for all strains, except FT65, (P<0.05).

To our knowledge, this is the first study to correlate genetic and serotypic differences for an enteric CV and link the genetic classification scheme to a biologically relevant measure. In general, a strong correlation between ReCV genotypes and serotypes was observed. In an earlier study by Zheng et al. (2006), the minimum aa difference detected between NoV genotypes was 14.26 %, while in a more recent study including newly described NoVs, a difference of 13 % was reported (Kroneman et al., 2013). The smallest aa difference observed in this study between ReCV serotypes (FT285 and the other two G1.2 strains) was 12 %. On the other hand, we identified cross-neutralization between strains with a 25 % aa difference. FT157 or FT499 (serotype 4) and FT65-specific hyperimmune sera were not generated in this study. Follow-up studies that include mAbs and more ReCV isolates and sera are critical.

While we were unable to evaluate whether protection against infection is serotype-specific in the rhesus macaque model, the implication of our findings for human NoV research, especially vaccine development, is significant. Further clarification of the mechanism of ReCV antibody neutralization is important for human NoV research. Meanwhile, since the VP1 sequences and cross-VN results disclose valuable information, we performed computational B cell epitope predictions on modelled 3D structures of ReCVs. A candidate epitope on loop A'-B' of the P2 domain was identified (Fig. 4) that was also predicted at a similar location on several human NoV structures (data not shown). A literature search
revealed that an equivalent region on loop A’-B’ of the murine NoV P2 domain has been identified as one of the contact sites for a neutralizing mAb (A6.2) (Katpally et al., 2008). This epitope structurally overlaps with the highly variable key blockade epitope A that is reported to change with new G2.4 human NoV emergence (Lindesmith et al., 2013) (Fig. 4c).

In summary, our findings clearly demonstrate the value of the ReCV model as a surrogate for analysing the diverse biological features of human noroviruses, and pave the way for detailed evaluation of enteric CV antibody neutralization and escape as well as the relationship with HBGA binding through cell culture-based, structural and in vivo studies.

**METHODS**

**Viruses and cell lines.** Genogroup I ReCVs were isolated from rhesus stool samples collected during our previous studies (Farkas et al., 2008, 2010a). All strains were isolated in LLC-MK2 cell lines (ATCC CCL-7). Virus stocks were prepared from viruses plaque-purified three times and stored at −80°C in aliquots.

**Anti-ReCV mouse hyperimmune sera.** Mice were maintained at the animal care facility of Cincinnati Children’s Hospital Medical Center (CCHMC), and all animal procedures complied with National Institutes of Health guidelines. Our animal protocol was approved by both the Institutional Animal Care and Use Committee of CCHMC (Animal Welfare Assurance Number A3108-01). Hyperimmune sera were generated against one representative strain of each genotype of G1 ReCVs, including the prototype TV (G1.1), FT285 (G1.2) and FT7 (G1.3). Three female 6- to 8-week-old BALB/c mice were injected intraperitoneally with 10^5–10^6 TCID_50 of CaG1 density gradient-purified ReCVs mixed with an equal volume of Sigma Adjuvant (Sigma Aldrich). Animals were boosted twice at 3-week intervals and anaesthetized/sacrificed via carbon dioxide (CO_2) inhalation on day 14 after the last immunization. Serum was collected and stored in aliquots at −80°C.

**RNA extraction and genome amplification.** Viral RNA was extracted with TRIzol LS (Life Technologies) according to the manufacturer’s instructions, resolved in 20 μl RNase- and DNase-free water and stored at −80°C. cDNA was synthesized using the SuperScript III First-Strand Synthesis System (Life Technologies) according to the manufacturer’s protocol, with a lock-docking oligo-dT primer containing a 5’ overhang [GGCCACGCGGTAGTACGGC(GT)AC(T)3 VN]. The 3’ portions of ReCV genomes (−3 kb) were amplified with gene-specific primers designed based on partial RNA-dependent RNA polymerase (RdRp) sequences described in our previous study (Farkas et al., 2010a) and the 5’ overhang primer, GGCCACGCGGTAGTACGGC, using the GoTag Green Master Mix (Promega).

**DNA sequencing.** PCR products were excised from 1.5% agarose gels, recovered using the Wizard SV Gel and PCR-cleanup system, and cloned into pGEM-T vector according to the manufacturer’s protocols (Promega). Positive clones were identified using PCR. Plasmid DNA was extracted from small-scale bacterial cultures using the Wizard Plus SV Miniprep DNA-purification system (Promega), according to the manufacturer’s instructions, and sequenced using M13 forward and reverse and gene-specific primers (primer walking) with the chain-termination method on an ABI PRISM 3730 DNA Analyser (Applied Biosystems). Each sample was sequenced in both directions from at least two independent clones. Consensus sequences were submitted to the GenBank database under accession numbers KC662363–KC662370 and KF431831.

**Sequence and phylogenetic analyses.** BLAST analyses were run against NCBI databases. Multiple sequence alignments of nt and aa sequences were created using Omega v2.0 software (Oxford Molecular). Phylogenetic trees were constructed using the unweighted pair group method with arithmetic mean (UPGMA) and the neighbour-joining clustering methods of the Molecular Evolutionary Genetics Analysis (MEGA version 5.1) software with Jukes-Cantor and Poisson correction distance calculations for nt and aa sequence alignments, respectively (Tamura et al., 2011). The confidence values of the internal nodes were obtained by performing 1000 bootstrap analyses. Accession numbers of the TV, WUHARV, Recovirus Bangladesh/289 and human NoVs included in the alignments are listed in Fig. 2a.

**Cross-titration of mouse hyperimmune sera.** A cytotoxic effect (CPE)-based virus neutralization assay was performed with heat-inactivated (56°C, 30 min) serum samples, as described previously (Farkas et al., 2010a, b, 2012). Briefly, twofold serial dilutions of hyperimmune antisera were titrated against 100 TCID_50 of TV, FT285 and FT7 ReCVs. The virus/serum mix was incubated for 1 h at 37°C and transferred to duplicate wells of 96-well tissue culture plates seeded with 10^4 LLC-MK2 cells per well. Virus and mock-inoculated control wells were included. Plates were stained with crystal violet at 72 h post-inoculation. By this time, all cells in the virus control wells were rounded and detached. End titrations were repeated in several experiments. End titres were calculated as mean values based on the highest dilution at which the cell monolayer was >50% intact in both wells. This dilution against the homologous ReCV strain was taken as one VNU. Similar to entero-, rhino- and rotavirus serotyping (Committee on Enteroviruses, 1962; Kapikian et al., 1967; Wyatt et al., 1982), a 20-fold or greater difference in VN titres was required in both directions for establishing a ReCV serotype.

**Serotyping plaque assay.** Due to the limited volume of the mouse hyperimmune sera (~0.5 ml/animal), serotyping of the 10 G1 ReCV isolates was performed in a cross-neutralization plaque assay with ~50 and ~100 p.f.u. of virus and 20 VNUs of each hyperimmune serum. A similar method was previously used for the serotyping of several viral agents, including avian infectious bronchitis viruses (Cowen & Hitchner 1975) and vesiviruses (Smith et al., 1998). Briefly, serum/virus mix and controls were incubated for 1 h at 37°C and adsorbed to LLC-MK2 monolayers in six-well tissue culture plates (Corning Life Sciences) for 1 h at 37°C. Plates were washed twice with PBS and overlaid with culture medium containing 1.2% Avicol (FMC BioPolymer). After 72 h of incubation, plates were stained with crystal violet and plaques counted. Each assay was repeated at least twice. ReCV strains with ≥80% of plaque reduction were considered to be neutralized. Strains neutralized by a single specific antiserum were grouped as a serotype.

**Virus neutralization epitope prediction.** Computational prediction of linear B cell epitopes was performed with BepiPred 1.0 (Larsen et al., 2006). Three-dimensional structures of ReCV capsid proteins were generated using the Phyre 2 server (Kelley & Sternberg, 2009). Discontinuous B-cell epitopes were predicted with the DiscoTope 2.0 server (Kringlem et al., 2012). The predicted B-cell epitopes were evaluated based on analyses of multiple sequence alignments and cross-neutralization results (regions with serotype-specific differences) and the predicted three-dimensional structures (internal or surface-exposed regions) of ReCVs.

**Statistical analysis** Statistical significance of cross-VN titres among the ReCV isolates was evaluated with Fisher’s exact test.
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REFERENCES


