GENETIC DIVERSITY AND HISTO-BLOOD GROUP ANTIGEN
INTERACTIONS OF RHESUS ENTERIC CALICIVIRUSES

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ABSTRACT

Recently, we reported the discovery and characterization of Tulane virus (TV), a novel rhesus calicivirus (CV). TV grows well in tissue culture, and represents a new genus within *Caliciviridae*, with the proposed name of “Recovirus”. We also reported a high prevalence of CV antibodies in macaques of the Tulane National Primate Research Center (TNPRC) colony, including anti-norovirus (NoV), anti-sapovirus (SaV) and anti-TV. To broaden our knowledge about CV infections in captive non-human primates (NHP), 500 rhesus macaque stool samples collected from breeding colony TNPRC macaques were tested for CVs. Fifty seven (11%) samples contained recovirus isolates. In addition, one NoV was detected. Phylogenetic analysis classified the recovirus isolates into two genogroups and at least four genetic types. The rhesus NoV isolate was closely related to GII human NoVs. TV neutralizing antibodies were detected in 88% of serum samples obtained from primate care-takers. Binding and plaque reduction assays revealed the involvement of type A and B histo-blood group antigens (HBGA) in TV infection. Taken together, these findings indicate the zoonotic potential of primate CVs. The discovery of genetically diverse and prevalent group of primate CVs and remarkable similarities between rhesus enteric CVs and human NoVs opens new possibilities for research involving *in vitro* and *in vivo* models of human NoV gastroenteritis.
INTRODUCTION

Caliciviruses (CV) are important human and animal pathogens causing a wide variety of diseases in their respective hosts. The family *Caliciviridae* consists of five established genera (*Norovirus, Sapovirus, Lagovirus, Vesivirus, and Nebovirus*). Recently, two new calicivirus genera have been proposed, represented by the Tulane virus (“Recovirus”) and the St. Valerien-like viruses (“Valovirus”) (11–13, 23, 36, 37, 39).

NoVs are recognized as the leading cause of epidemics of gastroenteritis (GE), causing 80–90% of nonbacterial GE outbreaks and over 50% of all food related GE outbreaks (7, 8, 29). They are also an important cause of sporadic GE in both children and adults.

Based on phylogenetic analysis, NoVs are divided into 5 genogroups and over 30 genetic clusters or genotypes (9, 46). This high genetic and likely antigenic variation combined with the lack of a tissue culture or animal model represent major obstacles for NoV research.

NoVs with close genetic and antigenic relatedness to human NoVs have been isolated from various animal species (6, 28, 33, 41). This not only provided opportunities for using some of these viruses as surrogates for human NoV research (44), but has also raised the concern about the possible zoonotic nature of CV gastroenteritis.

Based on results of *in vitro* binding assays, volunteer challenge studies and analysis of NoV outbreaks, it was proposed that histo-blood group antigens (HBGA) including the ABO, Lewis and secretor type HBGAs may function as the NoV receptors (17, 19, 20, 27, 32). Involvement of other host factors in NoV replication and susceptibility to infection has also been implicated (14, 43).
Previously, we reported the isolation and characterization of a novel CV (Tulane virus; TV) from stool samples of juvenile rhesus macaques (11). TV represents a newly proposed genus (Recovirus) within Caliciviridae that phylogenetically shares a common origin with NoVs; however, TV can be grown in tissue culture (11). We also reported a high prevalence of anti-NoV, anti-SaV binding and anti-TV neutralizing (VN) antibodies in colony macaques, suggesting that CV infections are frequent in captive non-human primates (NHP) (10). The few NoV challenge studies conducted also suggest that NHPs are susceptible to NoV infection. Chimpanzees inoculated with the Norwalk virus developed seroresponses and virus shedding but without the manifestation of clinical disease (45). Subekti et al. reported the development of clinical illness characterized by diarrhea, dehydration, vomiting and virus shedding in newborn pigtail macaques inoculated with the Toronto virus (40). In a study conducted by Rockx et al., one of the three rhesus macaques infected with the Norwalk virus developed virus-specific IgM and IgG responses and shed the virus for 19 days post inoculation (38). To date, however, direct evidence of natural NoV or SaV infection in NHPs is missing. Moreover, the prevalence and genetic diversity of recoviruses have yet to be studied.

In this study, we undertook the molecular detection and genetic analysis of CVs circulating in colony macaques and examined the role of HBGAs in recovirus infection.

MATERIALS AND METHODS

Sample collection. Stool, saliva and serum samples were collected between April and July of 2008 from 500 randomly selected juvenile (<3-years-old) rhesus macaques (Macaca mulatta) of Indian origin during the semiannual TNPRC breeding colony.
inventory. A rectal loop stool sample was collected from each animal and ~20% (w/v) of stool suspensions were prepared in phosphate-buffered saline, pH 7.4 (PBS). Saliva samples were collected by cotton-tipped applicators which were immersed into 0.5 ml sterile PBS after sampling. Four ml of EDTA blood was obtained from each macaque and serum was harvested. Samples were aliquoted and stored at -80 °C.

All samples were obtained in accordance with the standards of the Association for Assessment and Accreditation of Laboratory Animal Care. Only simian immunodeficiency virus, simian retrovirus, simian T-cell leukemia virus, and Cercopithecine herpesvirus (B virus) negative animals were used. Investigators adhered to the Guide for the Care and Use of Laboratory Animals prepared by the National Research Council. NHP samples were handled in compliance with BSL2+ laboratory practices approved by the Institutional Biosafety Committees of the participating institutions.

Saliva samples were collected from adult human volunteers at Cincinnati Children’s Hospital Medical Center, following a protocol approved by the Institutional Review Board.

**Virus stock.** In this study to keep mutations at the lowest level, TV stock was prepared from plaque purified TV at the 3rd passage. Virus stock was aliquoted and stored at -80 °C.

**RNA extraction and RT-PCR.** Viral RNA was extracted by the QIAamp viral RNA mini kit on a QIAvac 24 plus vacuum manifold (Qiagen Inc., Valencia, CA) according to the manufacturer’s instructions. Twenty two stool samples along with negative (deionized water) and positive (cell culture-adapted TV of 10^4 pfu/ml) controls were
extracted at a time. Extracted RNA was eluted into 30 µl buffer and stored at -80 °C. CV-specific RNA was amplified from 3 µl of extracted RNA template using the AccessQuick RT-PCR system (Promega, Madison, WI) according to manufacturer’s instructions with modified P289/P290 primers (9, 22), targeting the RNA-dependent RNA polymerase region (RdRp). These primers produce 313 bp, 319 bp and 331 bp products for TV, NoV and SaV, respectively. RT-PCR conditions were as described previously (11).

DNA sequencing. RT-PCR products were excised from agarose gels, recovered by the QIAquick gel extraction kit (Qiagen Inc., Valencia, CA) and cloned into pGEM-T vector (Promega, Madison, WI) according to the manufacturers’ protocols. Positive clones were identified by PCR. Plasmid DNA was isolated from 2 ml cultures by the QIAprep spin miniprep kit (Qiagen Inc., Valencia, CA) according to the manufacturer’s instructions and sequenced using M13 forward and reverse primers by the chain termination method on an ABI PRISM® 3730 DNA Analyzer (Applied Biosystems Inc, Foster city, CA). Each sample was sequenced in both directions from two independent clones.

Phylogenetic analysis. Multiple sequence alignments of partial RdRp nucleotide sequences were created using the Omiga v2.0 software (Oxford Molecular Ltd, Oxford, UK). Dendrograms were constructed by the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) and the Neighbor-Joining clustering methods of the Molecular Evolutionary Genetics Analysis (MEGA version 3.1) software with Jukes-Cantor distance calculations. The confidence values of the internal nodes were obtained by performing 1025 bootstrap analyses. The following GenBank sequences were used in the analysis: Boxer (AF538679), Chiba (AB042808), Desert Storm (U04469), Gifu96 (AB045603), Hawaii (U07611), Hesse (AF093797), Hokkaido/83(AB231342), Lordsdale
Virus neutralization (VN) assay. A cytopathic effect (CPE)-based VN assay was performed with heat-inactivated (50 °C, 30 min) serum samples and 100 TCID\(_{50}\) of the prototype TV as described previously (10). All rhesus serum samples were tested in duplicate wells, with two-fold dilutions starting from 1:10. In addition to rhesus serum samples, 100 archived human serum samples collected from TNPRC animal caretakers in the 1990’s were also tested starting from 1:2 dilution.

Confirmation of NoV in a rhesus macaque stool sample. Based on the NoV sequence (FT244) obtained from the macaque stool sample with P289/P290, one forward (P121/131F 5’- CCAGCTTGATGTAGGCGATT -3’) and two reverse (P121R 5’- CTCAGCCATTGCACCTCAAAG -3’ and P131R 5’- GACCTGACACCTCAGCCATT -3’) strain specific primers were designed. These primers produced a 121 and a 131 bp amplicon, respectively, within the RdRp region. In order to further corroborate the presence of NoV in the macaque stool and to eliminate the possibility of sample contamination at CCHMC, two additional aliquots of the original sample were retested at the TNPRC using the strain specific primers.
HBGA phenotyping. Phenotyping of rhesus and human saliva samples was performed by ELISA using commercially available monoclonal antibodies (MAbs) specific to ABH and Lewis (Le) antigens as previously described (17). MAbs used in this study included: anti-A (ABO1), and anti-B (ABO2) (Diagast, Loos, France), anti-H type 1 (BG-4), anti-Le\(^a\) (BG-5), anti-Le\(^b\) (BG-6), anti-Le\(^x\) (BG-7) and anti-Le\(^y\) (BG-8) (Covance, Berkeley, CA). Rhesus saliva samples recovered from swabs were tested at a 1:25 dilution, human saliva samples were tested at a 1:500 dilution. All samples were tested in duplicate wells.

HBGA binding. Twenty four human saliva samples (eight each of A, B and O type) and type A and type B trisaccharides conjugated to bovine serum albumin (BSA) (Glycorex AB, Lund, Sweden) or to biotin-labeled polyacrylamide (PAA) (GlycoTech, Gaithersburg, MD) were use to evaluate the HBGA binding properties of the prototype TV, as described previously for NoV virus like particles (VLPs) (17).

Briefly, ELISA plates (Corning Life Sciences, Lowell, MA) were coated with boiled human saliva samples at a 1:500 dilution, or with BSA-conjugated type A or B trisaccharides (1 \(\mu\)g/well) overnight, at 4 ºC. To coat the biotin-labeled PAA-conjugated type A and B trisaccharides, ELISA plates were pre-coated with Neutravidin (1 \(\mu\)g/well) (Thermo Fisher Scientific Inc., Waltham, MA) for 3 hours at room temperature, blocked with 5% skimmed milk-PBS and incubated with the biotin-PAA- type A or B trisaccharides (1 \(\mu\)g/well) overnight at 4 ºC. All plates were blocked with 5% skimmed milk-PBS and 10\(^{5}\) TCID\(_{50}\)/well purified TV in 1% skimmed milk-PBS was added. Saliva or synthetic oligosaccharide coated wells without TV served as negative controls. Plates were incubated for 2 hours at 37 ºC and virus binding was detected by hyperimmune anti-TV mouse sera and HRP-conjugated goat anti-mouse IgG,A,M (H+L) (Invitrogen,
Carlsbad, CA). Samples were analyzed at least in two separate experiments. TV binding was established based on a P/N ratio > 2 and an OD value ≥ mean of negative controls plus 2 SD.

Plaque reduction. Saliva samples (n=24) at a 1:100 dilution or type A and B synthetic oligosaccharides (0.1-10 µg/ml) were mixed with 50 – 100 plaque-forming units (PFU) of TV and incubated for 1 hour at 37 ºC. The following synthetic oligosaccharides were tested: BSA-conjugated type A and B trisaccharides (Glycorex AB, Lund, Sweden), PAA-conjugated type A and B trisaccharides (GlycoTech, Gaithersburg, MD) and A-Leb-pentasaccharide and B-Leb pentasaccharide (Sigma-Aldrich, St. Louis, MO).

Samples including virus controls were adsorbed to LLC-MK2 monolayers in 6 well tissue culture plates (Corning Life Sciences, Lowell, MA) for 1 hour at 37 ºC. Plates were washed twice and overlayed with M199 culture medium containing 0.8% methyl cellulose. Plates were stained with crystal violet on day 4 post-infection and plaques were counted. Each sample was tested in at least two wells in two separate experiments.

Blocking efficiency was calculated by 1 – (PFU of sample/ PFU of virus control) and expressed as percentage.

Statistical analysis. Statistical significance in binding and plaque reduction assays was calculated by two-tailed t-test with unequal variances, and P values < 0.05 were considered as significant.

RESULTS

Molecular detection of enteric CVs in colony rhesus macaques. Fifty eight (11.6%) of the 500 stool samples yielded CV-specific PCR product. All amplicons were cloned and
sequenced, revealing 57 recovirus (268 bp) and one NoV sequence (274 bp). The recovirus sequences exhibited 61-100% nt and 62-100% aa homology with the prototype TV (EU391643). The NoV sequence (FT244) revealed the highest nt homology (94%) with human NoV isolates EU072307 and EU007752 of the GenBank depository.

**Confirmation of NoV in rhesus macaque stool.** Amplification of the NoV specific sequence from rhesus macaque stool sample was confirmed by repeated detection and sequencing at CCHMC. Since the CCHMC laboratory routinely handles human NoV samples, it was necessary to exclude the possibility of laboratory contamination. Therefore, aliquots of the original stool sample that were kept at the TNPRC were tested with NoV-specific primers, yielding identical sequences to those detected at CCHMC, thus confirming the authenticity of rhesus NoV strain FT244.

**Phylogenetic analysis.** Phylogenetic analysis clearly divided the 57 recovirus isolates into four distinct groups, suggesting the existence of at least four recovirus genotypes within two genogroups (Figure 1). Among the 57 isolates 15 (26%), 11 (19%), 25 (44%), and 6 (11%) grouped to GI.1, GI.2, GI.3, and GII.1, respectively. The mean intergenogroup distances between the GI and GII recoviruses (0.536 - 0.613) were comparable to distances between the GI and the GII NoVs (0.534 - 0.695) (Table 1). Similarly, the mean distances between the recovirus genotypes within GI (0.251 - 0.359) were comparable with distances between the GI or GII NoV genotypes (0.289 - 0.352 and 0.251 - 0.346, respectively).

Phylogenetic analysis consistently placed the rhesus NoV isolate (FT244) to a branch rooting together with GII/6, GII/7 and GII/9 NVs (Figure 2). Multiple sequence alignments and distance calculations revealed 90%, 93% and 89% homology scores and
distances of 0.088, 0.058 and 0.073 between FT244 and the GII/6, GII/7 and GII/9 NoV sequences used in the alignment, respectively.

Rhesus enteric CV infection and diarrhea. Among the 500 breeding colony juvenile animals, selected for this study, 101 (20%) exhibited clinical signs of diarrhea at any time within a one-month period prior of sample collection. Eleven of the 57 (19%) recovirus positive animals had clinical history of watery diarrhea during the same period. Since diarrhea can be caused in NHPs by a variety of causes, and most of the animals in this study possessed TV- neutralizing antibodies, a controlled (experimental challenge) study with pre-selected seronegative animals is needed to corroborate or disprove the association between recovirus infection and clinical disease. Preparations for such an experiment are currently in progress.

TV-neutralizing antibodies and infection status. Serum samples collected at the time of the stool sample collection were available from 7 of the 15 GI.1, 10 of the 11 GI.2, 15 of the 25 GI.3, and 2 of the 6 GII.1 recovirus infected animals. VN titers against the prototype TV (GI.1) ranged from ≤10 to 160 in GI.1, from 20 to ≥1280 in GI.2, from 20 to 640 in GI.3 and from 40 to 640 in GII.1 recovirus infected animals. The mean VN titer was lower in animals infected with GI.1 viruses compared to animals infected with other genotypes. Although statistically significant differences in VN titers were observed only between GI.1 and GI.3 infected animals (Figure 3), such differences between genotypes may indicate homotypic antibody protection and the existence of serotypes.

TV neutralizing antibodies in humans. Overall, 88% of the 100 human serum samples tested neutralized the prototype TV at ≥1:2 dilution, indicating the potential for zoonotic transmission of TVs. The distribution of end titers was as follows: 1:256 (2%), 1:128
HBGA phenotypes and infection status. Saliva-based ABO phenotyping of the 500 animals revealed that these animals represented an almost homotypic population. Four hundred and eighty five animals (97%) secreted the B antigen (type B), 2 animals (0.4%) secreted both A and B antigen (type AB) and 13 animals (2.6%) had no detectable level of A or B antigen in their saliva (type O). Eleven of the 13 O type rhesus saliva contained the Lewis b (Le$^b$) antigen and all 13 were negative for the Lewis a (Le$^a$) antigen indicating that all, or perhaps all but 2 of the animals in this study were secretor type.

Among the 57 macaques that shed virus in their stools, 55 (96%) were type B and 2 (4%) were type O. One of the type O animals was infected with a GI.1 (FT218) and the other with a GI3 (FT269) virus. In order to evaluate the possible association between Lewis types and infectious status, the 57 recovirus positive animals, along with the 13 type O animals and 60 randomly selected VN antibody-negative animals were typed for Le$^a$, Le$^b$, Le$^x$ and Le$^y$ antigens. None of the animals expressed detectable levels of Le$^a$ antigen. The distribution of Le$^b$, Le$^x$ and Le$^y$ antigens was comparable among all the animals regardless of infection status (infected vs. VN negative). Furthermore, no difference in the distribution of Lewis antigens was found between animals infected by different genotypes (Table 2). Due to the homotypic distribution of HBGA in the subset of 500 macaques tested, recovirus infection could not be linked to a particular HBGA type, although both type B and type O macaques were infected with recoviruses. Future studies in macaques possessing more heterogeneous HBGA distribution are needed to determine
a potential association between HBGA phenotypes and susceptibility to recovirus infection in a population based study as described for NoVs (3, 24, 35).

**Saliva HBGA binding patterns of the prototype TV.** The prototype TV bound to 6 of the 8 type A and 7 of the 8 type B saliva samples. None of the type O saliva samples exhibited binding (Figure 4A). No association of binding with Lewis antigens could be established in any of the three groups. The eight type O saliva samples represented all four Lewis types screened for in this study.

**Synthetic oligosaccharide binding of the prototype TV.** Both BSA- or PAA-conjugated type A and B synthetic oligosaccharides were recognized by the corresponding anti-A (ABO1) or anti-B (ABO2) (Diagast, Loos, France) MAbs. OD values were comparable with those of saliva samples (OD 1.5-2) suggesting that both conjugated oligosaccharides contained the corresponding sugar moieties and were efficiently coated onto the plates. Nevertheless, TV binding was observed only with BSA- conjugated type A and B trisaccharides but not with PAA-conjugated ones (Figure 4B).

**Saliva plaque reduction assays.** In agreement with the saliva binding assays, both type A and type B saliva samples caused significant plaque reduction, while type O samples had no effect (Figure 5), regardless of the Lewis type. The type A, B and O saliva samples had 7-79%, 50-90% and 0-7% plaque reduction effect, respectively. Since variation among the virus control wells was ~10%, reductions of ≤ 10% were not considered. Similar to the binding assays an association between plaque reduction and any of the Lewis types tested was not evident.
Synthetic oligosaccharide plaque reduction assay. The pentasaccharide structures (ALeb and BLeb) and the PAA-conjugated trisaccharides exhibited no effect on the plaque numbers at any of the concentrations tested (0.1-20 µg/ml) (data not shown). The BSA-conjugated type A or B trisaccharides had no effect at concentrations lower than 1 µg/ml. However, a dose dependent 2 to 12 fold increase in plaque formation was observed at higher concentrations, starting at 2.5 µg/ml with the BSA-conjugated type B and at 7.5 ug/ml with the type A trisaccharide, respectively (Figure 6).

DISCUSSION

Recently, we reported the high prevalence of NoV-, SaV- and TV-specific antibodies in captive rhesus macaques(10). Although the existence of cross-reactive binding antibodies between human NoVs and the prototype TV suggested that anti-NoV antibodies detected in NHPs (10, 21) might be a consequence of TV infections, there is also evidence suggesting that NHPs are susceptible to at least experimental NoV infection (38, 40, 45). In this study, we targeted the molecular detection of CVs in TNPRC rhesus macaques with a generic primer set (P289/P290) that has been previously used for the detection of a variety of CVs (11, 15, 22, 23, 33). A high prevalence of recoviruses was detected with 57 (11.6%) of the 500 stool samples containing viruses closely related (62-90% nt homology) to the prototype TV. According to phylogenetic analysis, the recovirus strains detected in this study can be grouped into four genetic types within two genogroups. Of the 57 isolates, 15 (26%), 11 (19%), 25 (44%), and 6 (11%) grouped to GI.1, GI.2, GI.3, and GII.1, respectively (Figure 1). Such genetic diversity of recoviruses and circulation of genetically distinct strains in the TNPRC colony resembles the features of human NoVs. All the recoviruses detected in this study exhibited at least 10% nt difference when
compared to the prototype TV, which was isolated from stool samples collected in 2004 from the same colony (11). Existence of homologous antibody protection and existence of distinct serotypes that are likely associated with genotypes were suggested by the high prevalence (69%) of TV (GI.1) neutralizing antibodies in the colony (herd immunity) and by the higher TV neutralizing antibody titers in animals shedding viruses of heterologous (other than GI.1) genotypes (Figure 3). Consequently, tissue culture adaptation of the different recovirus strains, identification of serotypes, assessment of protective level of VN antibodies, T- and B-cell epitope mapping, and relation of antigenic epitopes to carbohydrate binding sites will provide valuable information for NoV vaccine design and development.

Interestingly, in one rhesus macaque stool sample a NoV was detected. Susceptibility of NHPs to experimental NoV infection was demonstrated previously (38, 40, 45). Nevertheless, this report is the first to describe natural NoV infection of a NHP. Laboratory contamination was excluded by repeated detection of the NoV from different aliquots of the original sample in two collaborating laboratories. Since stool samples were collected by individual rectal loops, contamination during sample collection could be excluded. The rhesus NoV isolate exhibited a 89-93% nt homology with GII/6, GII/7 and GII/9 human NoVs (Figure 2). Since separation of these genotypes in the RdRp region analyzed is poor, capsid-based (ORF2) sequence analysis of strain FT244 will be necessary to determine its exact genotype. Nevertheless, data generated in this study clearly placed the rhesus NoV isolate in a close proximity with human NoV isolates indicating the possibility of interspecies transmission. Despite the high prevalence of anti-NoV and anti-SaV antibodies in the TNPRC colony (10), only one NoV-infected
animal was detected in this study and no SaV was found. Whether the low detection rate of NoVs and SaVs compared to the high seroprevalence is due to the seasonal occurrence of these viruses in colony macaques, the unfitness of the primers or other reasons, remains to be elucidated. The possible zoonotic nature of enteric CV infections is also suggested by the high prevalence of TV neutralizing antibodies in serum samples of animal caretakers and the involvement of HBGAs in TV infection. The end titers of human samples were lower than the end titers of NHP samples. However, these human samples were collected nearly 20 years ago and antibody titers could be reduced during long-term storage. Parallel investigation of the epidemiology of enteric CVs in human and NHP populations therefore would be of interest.

Based on results obtained from in vitro binding assays and volunteer challenge studies, HBGAs have been identified as the putative cellular receptors for human NoVs (16, 17, 19, 20). The association between the HBGA binding abilities of NoVs and susceptibility to infection was established for some strains (27), however, for others, such a link was not found (26), indicating that different NoVs may use different strategies to establish infection and possible other factors than HBGAs might also be involved. Since there is no effective tissue culture or animal model available to study this relationship, the potential involvement of HBGAs in recovirus infection was examined in this study.

Despite the fact that NHPs and humans have four phenotypes of ABO blood groups (A, B, O and AB), only humans and the anthropoid apes express the ABH antigens on the surface of red blood cells (1). Both new and old world monkeys secrete ABH antigens in their saliva and possess anti-A and/or anti-B antibodies in their sera. Thus, ABO blood groups of rhesus macaques can be determined by detection of A/B antigens in saliva or
antibodies in serum. In this study, based on reported involvement of ABO, Lewis and host secretor status in NoV infection (16, 17, 20), saliva based assays were used for HBGA phenotyping of rhesus macaques. All commercially available MAbs used were confirmed to recognize the corresponding HBGAs antigens except the anti-H type I (BG-4) antibody, which showed no reaction with any of the human saliva samples, even at a 1:10 dilution.

Saliva-based ABO phenotyping of the 500 animals used in this study revealed an almost homotypic population with 97% of the animals being type B. These results created difficulty in linking or excluding any particular ABO phenotype to susceptibility to recovirus infection. Similarly, no association could be established for a particular Lewis phenotype. Among the 57 recovirus positive macaques, 55 (96%) were type B and 2 (4%) were type O. Recovirus infection of type O animals was also indicated by the presence of VN serum antibodies. Thus, at least both type B and type O macaques are susceptible to recovirus infection. To assess the role of type A antigen and the possible differences among the different strains in population based studies, macaques with more polymorphic distribution of the ABO types need to be tested.

Our findings are consistent with previous studies that reported group B as the major blood type (~97%) found in rhesus macaques (25, 34). On the other hand, Malaivijitnond et al., described polymorphism of the ABO blood group in rhesus macaques in Thailand although, variation among troops from different geographic locations was significant (30). Since ABO typing is not included in routine assessment of research colony macaques, identification of a colony with polymorphic HBGA distribution may pose a challenge.
In saliva binding assays, the prototype TV bound to both type A and B saliva but not to type O saliva (Figure 4A). The type A and type B binding was confirmed by binding of the prototype TV to BSA-conjugated type A and B trisaccharides (Figure 4B). Interestingly, no binding to the PAA-conjugated type A or B trisaccharides was observed even though the anti-A (ABO1), and anti-B (ABO2) MAbs recognized both the corresponding BSA- and PAA-conjugated trisaccharides. Similar discrepancies have previously been noted for NoV VLP binding (17, 18). Huang et al., demonstrated that while specific MAbs recognize the HBGAs present in human milk or saliva regardless of the carrier molecule, binding of NoV VLPs depends on both the specific carbohydrate structure and the carrier molecule (18). Marionneau et al., suggested that optimal binding of NoVs to the carbohydrate ligands may require an optimal density of the ligand, and binding may disappear at low densities (31). Since PAA-conjugated trisaccharides with various carbohydrate : PAA ratios were not available for our study this possibility remains to be elucidated.

Both previous observations with NoV VLPs and our experiments with TV indicate that the nature of carrier molecules play a critical role in the binding of enteric CVs to carbohydrate structures. HBGA binding interfaces of different NoV strains have been characterized by co-crystallizing NoV P domain dimers with tri- and pentasaccharides (2, 4, 5). In our study, there was no demonstrable interaction between the prototype TV and unconjugated type A and B pentasaccharide structures or PAA-conjugated type A and B trisaccharides. This indicates that interaction between enteric CVs and HBGAs is more complex than is described in these structural studies and future studies with more complex HBGA structures are necessary.
Results of plaque reduction assays confirmed the involvement of the type A and B HBGAs in recovirus infection. Preincubation of the prototype TV with type A or B saliva samples resulted in a significant reduction of plaque numbers (Figure 5). Type O saliva samples, the PAA-conjugated type A and B trisaccharides and ALe\textsuperscript{b} and BLe\textsuperscript{b} pentasaccharides had no effect in plaque assays (data not shown). Contrary to our expectation, both BSA-conjugated type A and B trisaccharides increased the plaque numbers by 2 to 12 fold in a dose dependent manner. This effect started at a lower concentration with the BSA-conjugated type B (2.5 µg/ml) compared to the type A (7.5 µg/ml) trisaccharide, indicating a higher affinity interaction with the prototype TV.

These observations create several controversies. According to the ability of saliva samples to block TV infectivity in vitro, saliva should act as an antiviral during natural infection. It is also difficult to explain how, contrary to saliva samples, BSA-conjugated synthetic oligosaccharides increased TV infectivity. One possible explanation can be the involvement of co-receptor/receptor interactions in TV infection. We hypothesize that attachment to the HBGA (co-receptor) is required to prime TV – possibly through conformational changes – to enable interaction with a yet unknown cell surface molecule (receptor). In saliva, HBGA structures are attached to a wide variety of molecules including high molecular weight mucins. It was shown that NoV VLPs bind to HBGA structures attached to high molecular weight, but not to HBGAs attached to low molecular weight carriers present in human milk and saliva (18). These large carriers of HBGAs, possibly due to stearic hindrance, interfered with receptor binding when saliva was used in our TV plaque assays. On the other hand the smaller BSA molecules had no interference with the receptor binding site. Assuming that TV receptor and co-receptor
molecules need to be in physical proximity to support optimal attachment and entry, isolated receptor molecules far from the co-receptor will not be able to support virus attachment and entry. In the case of HBGA-primed TV virions, these isolated receptor molecules alone could be sufficient which will lead to increased efficiency of infection and plaque numbers.

The involvement of co-receptor/receptor interactions in enteric CV infections is also supported by previous studies showing that cell surface expression of HBGAs alone does not confer susceptibility to NoV infection, even in cell types that are able to support the intracellular steps of NoV replication (14, 43).

Finally, the discrepancy with saliva acting as natural antiviral in vitro could be explained by a yet unknown mechanism (pH, digestive enzymes) that in the stomach or small intestine dissociates the HBGA-mucin complexes or otherwise digests the large carrier structures, thus exposing the receptor interaction sites.

In summary, this study describes the high prevalence of genetically diverse rhesus enteric CVs, in conjunction with evidence that suggests the zoonotic potential of enteric CV infections and the involvement of HBGAs in recovirus infection. Although more detailed studies of recovirus HBGA interactions are necessary, including the characterization of the different isolates described in this study and in vivo studies to link HBGAs to susceptibility to recovirus infection, the data presented here, together with our previous reports (11, 42), collectively indicate the potential that recoviruses can be used to address research questions applicable to NoV gastroenteritis.

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LITERATURE


Table 1. Phylogenetic distance scores between Recovirus and Norovirus genetic types.

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<td>GI.3</td>
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<td>0.321</td>
</tr>
<tr>
<td>GII.1</td>
<td>0.536</td>
<td>0.540</td>
</tr>
<tr>
<td>GI.1</td>
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<tr>
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<tr>
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<tr>
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<tr>
<td>GII.3</td>
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Between group average phylogenetic distances were calculated based on nucleic acid sequence alignments of the corresponding partial RdRp region of recoviruses (268 bp) and noroviruses (274 bp). Group assignments of recovirus isolates were based on Figure 1. Norovirus genogroups were represented by the Norwalk (G1.1), Southampton (G1.2), Desert Shield (G1.3), Hawaii (GII.1), Melksham (GII.2) and Mexico (GII.3) viruses.
Table 2. Distribution of HBGAs in macaques assigned for this study.

All 500 animals were tested for ABO phenotypes. The recovirus positive animals (n=57) and 60 animals without detectable levels of VN antibodies against the prototype TV were also tested for Lea, Leb, Lex and Ley. Saliva based ELISA test using specific MAb was used. GI.1 –GI.3 and GII.1 indicate recovirus genotypes.

<table>
<thead>
<tr>
<th>Colony (n=500)</th>
<th>A</th>
<th>B</th>
<th>AB</th>
<th>O</th>
<th>Lea</th>
<th>Leb</th>
<th>Lex</th>
<th>Ley</th>
</tr>
</thead>
<tbody>
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<td>485</td>
<td>2</td>
<td>13</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
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<td>(64%)</td>
<td>(45%)</td>
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<td>(83%)</td>
<td>(50%)</td>
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</tr>
<tr>
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<td>0</td>
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<td>55</td>
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<tr>
<td>(n=57)</td>
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<td>(75%)</td>
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<tr>
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<td>31</td>
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</tr>
<tr>
<td></td>
<td>(97%)</td>
<td>(3%)</td>
<td></td>
<td>(70%)</td>
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<td>(70%)</td>
<td>(52%)</td>
<td>(100%)</td>
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</tr>
</tbody>
</table>
FIGURE LEGENDS

Figure 1. Rhesus enteric caliciviruses are genetically diverse and can be classified into four genetic types within the two genogroups. Dendrogram was constructed by the Neighbor-Joining clustering method of the Molecular Evolutionary Genetics Analysis (MEGA version 3.1) software with Jukes-Cantor distance calculations. The confidence values of the internal nodes were obtained by performing 1,025 bootstrap analyses. The prototype Tulane virus (M33) isolated in 2004 from the TNPRC rhesus colony is circled.

Figure 2. The rhesus NoV isolate is closely related to human NoV isolates. Dendrogram based on alignment of 274 nt RdRp sequences was constructed by the Neighbor-Joining clustering method of the Molecular Evolutionary Genetics Analysis (MEGA version 3.1) software with Jukes-Cantor distance calculations. The rhesus NoV (FT244) isolate is circled.

Figure 3. Mean VN titers against the prototype TV (GI/1) in macaques with virus shedding. Animals were grouped by the genetic types of the recovirus isolates. VN titers were higher in animals infected with GI/2, GI/3 or GII/1 strains than in animals infected with GI/1 strains. Statistical significance was calculated between GI.1 (test virus) and the other groups using two-tailed t-test with unequal variances. P values < 0.05 were considered as significant. Error bars represent SD. n = number of samples available for VN. *, P < 0.05.
Figure 4. Saliva and synthetic oligosaccharide binding of the prototype TV. Eight type A, type B and type O saliva samples (A) and BSA- or PAA-conjugated type A and type B trisaccharides (B) were tested, respectively. Mean OD values of separate experiments were calculated. Error bars represent SD. ***, P < 0.0005; **, P < 0.01.

Figure 5. Saliva blocking of TV replication. Type A and type B saliva samples but not type O saliva samples blocked TV replication. Eight type A, type B and type O saliva samples were tested, individually. C= virus control. ABO and Lewis types are indicated. Plaque reduction was calculated by 1 – (PFU of sample/ PFU of virus control) and expressed as percentage. The mean was calculated from the results of two independent tests with two different TV concentrations (~50 PFU and ~ 100 PFU). Error bars represent SD. ***, P < 0.0005; *, P < 0.05.

Figure 6. BSA-conjugated type A and type B trisaccharides increase TV plaque numbers in a concentration dependent manner. C= virus control. Fold increase/decrease was calculated by PFU of sample/ PFU of control. The mean was calculated from the results of two independent tests with two TV concentrations (~50 PFU and ~ 100 PFU). Error bars represent SD. P values indicate statistical significance. *, P < 0.05.
Figure 2.
Figure 4.

![Graph showing OD 450 values for BSA-A, BSA-B, PAA-A, and PAA-B for A and B samples.](image-url)
Figure 5.
Figure 6.

GalNAcα1-3Fucα1-2Galβ-O-spacer-BSA (type A)

Galα1-3Fucα1-2Galβ-O-spacer-BSA (type B)

Fold increase

µg/ml

*