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Genetic analysis of G12P[8] rotaviruses detected in the largest U.S. G12 genotype outbreak on record

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Abstract

In 2006–07, 77 cases of gastroenteritis in Rochester, NY, USA were associated with rotavirus genotype G12P[8]. Sequence analysis identified a high degree of genetic relatedness among the VP7 and VP4 genes of the Rochester G12P[8] strains and between these strains and currently circulating human G12P[8] strains. Out of 77 samples, two and seven unique nucleotide sequences were identified for VP7 and VP4 genes, respectively. Rochester strain VP7 genes were found to occupy the G12-III lineage and VP4 genes clustered within the P[8]-3 lineage. Six strains contained non-synonymous nucleotide substitutions that produced amino acid changes at 6 sites in the VP8* region of the VP4 gene. Two sites (amino acids 242 and 246) were located in or near a described trypsin cleavage site. Selection analyses identified one positively selected VP7 site (107) and strong purifying selection at 58 sites within the VP7 gene as well as 2 of the 6 variant sites (79 and 218) in VP4.

Keywords

rotavirus; USA; VP7 protein; VP4 protein; VP8*; vaccine

1. Introduction

Before widespread use of the 2 currently licensed rotavirus vaccines, Group A rotaviruses (RVA) caused approximately half a million deaths yearly among children younger than 5

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years globally (Parashar et al., 2009). In the U.S., deaths were not common but RVA was a significant cause of morbidity in young children (Payne et al., 2008). RVA, members of the *Reoviridae* family, possess a genome of 11 double-stranded RNA (dsRNA) segments that encode 6 viral structural proteins (VP1-VP4, VP6 and VP7) and 6 nonstructural proteins (NSP1-NSP6) (Estes, 2007). Surrounding the dsRNA are 3 protein layers, a central core (VP2), a middle protein layer (VP6), and an outer capsid layer composed of VP7 and VP4 proteins (Estes, 2007). The traditional dual classification of RVA is based upon serotype specificities and the sequence diversity of the 2 outer capsid proteins, VP7 (glycosylated, G-type) and VP4 (protease-sensitive, P-type) (Estes, 1996). At least 27 G genotypes and 37 P genotypes have been identified (Matthijnsens et al., 2011; Trojnar et al., 2013). Of all possible combinations, 5 strains (G1P[8], G2P[4], G3P[8], G4P[8] and G9P[8]) are associated with 80–90% of the RVA disease burden (Patel et al., 2011). However, 3 G12 strains, G12P[6], G12P[8], and G12P[9] have been emerging in many areas of the world (Iturriza-Gomara et al., 2011; Matthijnsens et al., 2010).

After RVA genotype G12 was first reported in 1987 in the Philippines (Taniguchi et al., 1990), it was detected only sporadically for more than 13 years (Das et al., 2003; Griffin et al., 2002; Pongsuwanna et al., 2002; Samajdar et al., 2006). Recently, it has re-emerged worldwide, becoming the sixth most prevalent RVA VP7 genotype (Rahman et al., 2007). Based on phylogenetic and phylodynamic analyses, G12 genotype strains have been subdivided into 4 lineages (Matthijnsens et al., 2010; Rahman et al., 2007). Lineage I and IV each contain only one strain, the prototype G12 strain and a porcine G12P[7] strain, respectively. Lineage II consists of G12P[9] strains from South America and Asia. Lineage III contains the majority of the currently known G12 strains, which are associated with P types P[6] or P[8].

In 2009, the World Health Organization (WHO) recommended 2 RVA vaccines, Rotarix® and RotaTeq®, for routine immunization of all infants (2009). The monovalent attenuated human RVA vaccine, Rotarix® (GlaxoSmithKline Biologicals, Rixensart, Belgium), contains the single most prevalent genotype (G1P[8]) and was developed to induce heterotypic immunity against a number of epidemiologically and clinically important strains (Ruiz-Palacios et al., 2006). In contrast, the pentavalent bovine-human reassortant vaccine, RotaTeq® (Merck & Co, Inc., Whitehouse Station, NJ) was developed to provide serotype-specific immunity against 4 common G types (G1–G4) and one common P type (P[8]) (Vesikari et al., 2006). Based on large phase III trials and post marketing surveillance studied, both the monovalent RotaTeq® and the pentavalent RotaTeq® vaccines provide protection against severe RVA disease from a wide variety of circulating strains in industrialized and developing countries (Patel et al., 2011).

The Centers for Disease Control and Prevention (CDC) currently supports RVA surveillance in the U.S. through the New Vaccine Surveillance Network (NVSN) (Payne et al., 2008). During the 2006–07 RVA surveillance season, the G12P[8] strain was detected in 69% (77 of 111) of RVA-positive samples from the NVSN surveillance site in Rochester, NY. Currently this RVA outbreak is the largest in U.S. history associated with genotype G12 (Payne et al., 2009). This overwhelming prevalence of G12P[8] RVA during that season was very unusual, especially because the G12 genotype was not detected in Rochester during the

previous 2005–06 or following 2007–08 RVA seasons (Payne et al., 2009). In addition, the hospitalization rates in Rochester during the 2006–07 season increased 3-fold for RVA disease when compared to the 2005–06 season (Payne et al., 2009). The aim of this study was to characterize the G12P[8] strains detected in Rochester during the 2006–07 outbreak at the genetic and protein structure level and to compare them to U.S. and worldwide wild-type G12 strains to better understand the evolution of this emerging genotype. In addition, we wanted to compare the antigenic regions of the outer capsid proteins of these G12P[8] strains against the cognate protein sequences of current RVA vaccines.

2. Materials and Methods

2.1. Samples and molecular methods

As a part of the NVSN surveillance network, active RVA surveillance was carried out in Rochester N.Y. USA during the 2006–2007 RVA season (Payne et al., 2009). Stool samples were screened for RVA antigen by enzyme immunoassay using the Premier™ Rotaclone® Rotavirus enzyme immunoassay kit (Meridian Diagnostics, Inc., Cincinnati, OH). RNA extraction, reverse transcription-polymerase chain reaction (RT-PCR) based genotyping of the outer surface proteins VP7 and VP4 gene, and sequencing were carried out as described previously (Hull et al., 2011). All sequences were obtained using dsRNA extracted directly from stool without isolation in cell culture.

2.2. Data analysis

Sequence chromatograms were edited, and overlapping sequence fragments were assembled using Sequencher 4.8 (Gene Codes Corporation, Inc., Ann Arbor, MI). The sequences were aligned using MUSCLE implemented in MEGA 5.05 software (<http://www.megasoftware.net/>). Modeltest was implemented in MEGA 5.05 to determine the optimal evolutionary model for the dataset, which was selected based on the value of the corrected Akaike information criterion (AICc). Phylogenetic relationships were inferred using PhyML version 3.01 (Guindon and Gascuel, 2003) as implemented through SeaView version 4 (Gouy et al., 2010). aLRT statistics were computed for phylogram branches (Anisimova and Gascuel, 2006). The distance between strains was calculated in MEGA using the p-distance model. Selection analyses using FEL statistics were performed using the Datamonkey webserver to identify selective pressures on individual codons of the VP4 and VP7 genes (Delport et al., 2010; Pond and Frost, 2005). Selection pressures were determined at $p = 0.1$. The distance between strains was calculated in MEGA using the p-distance model.

2.3. Nucleotide sequence accession numbers

VP7 sequences (for strains Ro4426 and Ro4434) and VP4 sequences (for strains Ro4426, Ro4439, Ro4462, Ro4487, Ro4500, Ro4501, Ro4532 and Ro4537) were submitted to GenBank under accession numbers JN711097 through JN711106.

3. Results

3.1. VP7 sequence analysis

The standard G-specific genotyping RT-PCR primer pool used in this study (Hull et al., 2011) lacked a G12 -specific primer, resulting in high number of G non-typeable strains by RT-PCR based genotyping. Thus, we sequenced all VP7 genes. The 77 VP7 sequences corresponded to nt 88–890 of the VP7 gene of reference strain Wa and are represented by 2 unique sequences, RVA/Human-wt/USA/Ro4426/2007/G12P[8] and RVA/Human-wt/USA/Ro4434/2007/G12P[8]. High nucleotide and amino acid sequence similarities among the Rochester G12 sequences (99.7–100% and 100%, respectively) as well as between the Rochester G12 sequences and worldwide characterized human G12-III lineage strains (97.8–99.7% and 99.1–100%, respectively) were observed. Among Rochester strains, a single silent nucleotide substitution at position 172 was identified in the VP7 gene; it divided the viruses into 2 sub-groups, Ro4426-like and Ro4434-like strains. Fifty one Ro-4426-like strains had G in position 172, while 26 Ro-4434-like strains had A. Maximum likelihood phylogenetic analysis indicated genetic relatedness of the VP7 gene of the Rochester strains with 3 G12-III lineage (Rahman et al., 2007) strains from Tennessee (2008), India (2005) and Germany (2008) and shared a lineage with an Indian strain from 2001 (Figure 1A).

3.2. VP4 sequence analysis

The 77 VP4 sequences corresponded to nt 43–850 of the VP4 gene of reference strain Wa. They are represented by 7 unique sequences. High nucleotide (99.6–100%) and amino acid (98.9–100%) identities were found among the Rochester G12P[8] sequences, which cluster within the P[8]-3 lineage (Maunula and von Bonsdorff, 1998) and share a sublineage with aforementioned strains from Tennessee (Figure 1B). Nucleotide and amino acid sequence identities with other P[8]-3 strains were 98.3% or higher. Sixty nine Rochester strains were identical to the Ro4426 VP4 gene (70 total), while another 7 strains exhibited 1 to 2 nucleotide differences (Figure 2). Strains Ro4532 is identical to Ro4439, both have been submitted to GenBank.

These few nucleotide changes in the VP4 gene of 7 Rochester G12P[8] strains (Ro4537, Ro4439, Ro4532, Ro4462, Ro4487, Ro4500, Ro4501) resulted in amino acid changes at 6 sites (Figure 2). Using the VP8* crystal structure of reference strain Wa (Blanchard et al., 2007), the changes mapped to the following sites: amino acid 79, located on the loop between 2 β -sheets, β -B and β -C (Ro4487); amino acid 104, located on the β -E sheet (Ro4439 and Ro4500); amino acid 199, located on the β -M sheet (Ro4462 and Ro4501); amino acid 218, located on an α -A helix (Ro4500); and amino acids 242 (Ro4537) and 246 (Ro4439 and Ro4500), located in the protease sensitive region of VP4 (Figures 2 and 3). All 6 of these sites are located outside the described antigenic region of VP8* (Kirkwood et al., 1996).

3.3. Selection analysis

Selection analysis of the G12 VP7 gene identified one positively selected site (L107M, $p = 0.027$) and strong purifying selection at 58 sites, ($0.0004 < p < 0.094$). The positively selected site is present in helix B (α B) of the protein, which is involved in the formation of

the trimeric VP7 structure (Figure 4). Although position 107 is not directly involved in the formation of the trimer surfaces, its close proximity to the surface may affect the affinity and/or strength of binding of VP7 to itself in the trimeric complex. Mutagenesis studies are required to test the effect of this change. It is important to note that the L107M change was not present in the G12 Rochester strains but was found in strains VU05–06–72 (G12P[8] from Tennessee, USA) and K12 (G12P[9] from Japan).

Selection analysis of P[8] VP4 gene sequences identified no sites under positive selection, but strong purifying selection was identified at 103 amino acid positions (0.0006 p 0.091). Out of the 6 sites that exhibited amino acid changes due to nucleotide changes in the VP4 gene, position 79 and position 218, which are found on the surface of the protein, were under strong purifying selection based on the FEL analysis ($dn/ds < 1$). In this study, strain Ro4487 exhibited a D79N change and a S79G substitution has been reported among the VP4 changes found in a strain SA11 neutralization escape mutant (Gorziglia et al., 1990). Ro4500 exhibited a Y218C substitution. Purifying selection at these sites suggests conservation of function and the observed substitutions may have no or minimal detrimental effect on the functioning of the protein (Soskine and Tawfik, 2010). This is also supported by the fact that these substitutions were found only in single strains within the entire population ($n = 77$).

4. Discussion

In this study, selection analysis of the VP8* region and the VP7 gene, large regions of purifying selection were identified for Rochester G12P[8] strains. The data suggest a conservation of structure and function in those regions of the proteins between all the strains used in this study. In the VP8* region two surface amino acids, 79 and 218, showed conservative amino acid changes in strains Ro4487 and Ro4500, respectively. As these sites were under strong purifying selection, we can assume, in the absence of experimental data, that these substitutions have very little effect on the protein function. Among strains used in this study for the phylogenetic analysis, one site in the VP7 gene (position 107) was identified as positively selected but the substitution was absent in all Rochester strains. The site was found to be present on helix B (Figure 4), which is involved in the VP7 trimer formation. Mutagenesis studies are further required to understand the role of this position in the trimeric complex. In this study we report the first instance of selection analysis on the VP8* region of the VP4 gene. Although there exists a report describing positive selection in VP7 gene at positions 119 and 199 (Song and Hao, 2009), this is the first time positive selection on position 107 was observed. It is interesting to note that the two positions 119 and 199 identified were positively selected in the bovine (G8) and canine (G3) lineages of rotavirus. Position 119 lies in the B2 sheet and is not involved in the trimeric formation. On the other hand position 199 lies in the loop structure between B7 and B8 sheets and is possibly involved in the VP7 trimer interface. The actual function of these positions has not yet been experimentally determined. The only other report of positive selection in rotavirus exists for the NSP2 gene where 4 sites were identified as positively selected (Donker and Kirkwood, 2012) although the functional relevance of these sites are yet to be determined.

The VP4 protein of most animal RVA strains contains 776 amino acids, whereas most human RVA strains lack a residue in the proximity of position 135. Thus, the VP8* region of most human strains is one residue shorter than the VP8* of animal strains (Estes, 1996). Based on the SA11 (simian strain) sequence, 2 trypsin cleavage sites R241 and R247, corresponding to R240 and R246 for human P[8] strains, have been identified (Lopez et al., 1985). For some human strains, a third trypsin cleavage site may also be present at position 230 which is either an arginine or lysine residue (Gorziglia et al., 1986). The aforementioned arginine residues are highly conserved in RVA strains independent of genotype, and cleavage at arginine 246 correlates with enhanced infectivity (Arias et al., 1996; Gilbert and Greenberg, 1998; Lopez et al., 1986). R230 and R240 are conserved among Rochester G12P[8] strains but 2 Rochester G12P[8] strains, Ro4439 and Ro4500, exhibit a conservative amino acid change, R246K, at site 246. Also, the VP4 of strain Ro4537 exhibits a conservative amino acid change in the trypsin cleavage region between the VP8* and VP5* proteins, I242V. This change may have an effect on infectivity of this RVA strain by altering the affinity of trypsin for the VP4 protein. All Rochester G12P[8] strains except for Ro4487 contain aspartic acid at position 79 in the VP8* region. An amino acid change of D79N is located on the loop between β B and β C sheets on the surface of the protein. The net effect of losing a negative charge in position 79 for strain Ro4487 is unknown.

The VP8* core protein fold resembles the basic structural features of galectins (Dormitzer et al., 2002). Many of the VP8* amino acid residues that anchor the β -ribbon (β F and β G, Figure 2) to the 6-stranded β -sheets are in positions equivalent to the galectin carbohydrate binding residues (Dormitzer et al., 2002). One of those residues is an alanine at position 104, and 3 strains, Ro4439, Ro4532 and Ro4500, have threonine instead of alanine at this position. Position 104 is located on the β E-sheet in a previously-defined variable region (Gorziglia et al., 1990) and possibly interacts with V121 on the β F-sheet or F122 on the loop between the β F-sheet and β G-sheet. Because threonine is one carbon longer and bulkier than alanine, this residue potentially can influence the position of the loop that contains F122 and is located on the surface of the protein. Presence of the hydroxyl group in the position 104 when threonine, a polar uncharged residue, is present instead of alanine, a hydrophobic residue, could potentially have an effect on interactions with V121 or F122. Two Rochester strains, Ro4462 and Ro4501 have a non-conservative change, T199I, which is located on the surface of the protein. Interestingly, RotaTeq® has an isoleucine at this site, while Rotarix® has threonine. In strain Ro4500, another non-conservative amino acid change, Y218C, also is located on the surface on the protein. The VP8* antigenic regions of the Rochester G12P[8] strains and RotaTeq® are almost identical with only one amino acid difference (Figure 2). In contrast, only 20 out of 25 residues in the antigenic region are conserved between the Rotarix® P[8] and Rochester G12P[8] strains, suggesting that RotaTeq® might confer better protection against these strains.

As expected, the G12 VP7 antigenic regions, including 7-1a, 7-1b, and 7-2 (Zeller et al., 2012) of Rochester G12 strains differ substantially from those of both Rotarix® and RotaTeq® VP7 (data not shown). For example, only 12 out of 29 amino acid residues are identical between Rochester G12 strain proteins and the G1 protein of Rotarix®. Among the RotaTeq® component strains, 9 to 15 amino acids in the antigenic region are conserved between the VP7 proteins of Rochester G12P[8] strains and RotaTeq® G1-G4 and G6

strains. Because the mechanisms of protective immunity to RVA infection are not fully understood and are believed to likely involve other RVA genes besides VP7 and VP4 (Desselberger and Huppertz, 2011), we cannot predict if the degree of amino acid identity between Rochester G12 strains and vaccine strains in the antigenic regions of VP4 and VP7 proteins correlates with vaccine effectiveness. Against G12P[8] strains circulating in the United States, Payne et al. (Payne et al., 2013) estimated the efficacy of RotaTeq® vaccine to be 83% but could not calculate a comparable effectiveness estimate for Rotarix® due to inadequate numbers of cases and controls.

RotaTeq® vaccination started in Rochester, NY in 2006 and coverage with the vaccine was estimated to be 42.5% by 2007 (Rotarix® was not licensed until 2008), the highest uptake at any of the 3 NVSN surveillance sites (Payne et al., 2009). While the number of hospitalizations due to RVA declined in Cincinnati and Nashville from 2006 to 2007, the number of cases in Rochester actually increased by 72% as a result of the G12P[8] outbreak (Payne et al., 2009). Based on their VP7 nucleotide similarities, newly emerged G12 strains are closely related to each other (>96.9% nt identity) but substantially divergent from early human G12 isolates, with < 91% nucleotide identity observed (Banyai et al., 2007). However, genetic analysis of all 11 gene segments, as well as electropherotype migration patterns of G12 strains, revealed a very large diversity and a high reassortment activity among the newly emerged G12 population (Rahman et al., 2007; Uchida et al., 2006). Full genome analyses revealed that recent G12-III strains (from 2002 and later) more commonly possess a Wa-like genetic backbone as opposed to DS-1-like or AU-1-like backbone (Freeman et al., 2009; Pietsch and Liebert, 2009; Rahman et al., 2007) and the presence of the Wa-like backbone is believed to have played a role in the worldwide spread of human G9 and G12 RVAs. The G12 genotype has increased in prevalence worldwide (Rahman et al., 2007) and is currently the most frequent “uncommon” strain detected in Europe and Australia (Iturriza-Gomara et al., 2011; Kirkwood et al., 2009). It has been hypothesized that the G12 genotype in combination with the Wa-like backbone might result in higher virulence and more efficient replication due to the evolutionary advantages of possessing a Wa-like backbone (Freeman et al., 2009) and this hypothesis is supported by the observed higher degree of severe disease associated with this outbreak (Payne et al., 2009).

One limitation of the study was that we did not sequence the VP5* portion of VP4 gene for the Rochester G12P[8] strains, a region that contains 5 additional antigenic regions because this region was not included in the amplicon generated by the con2/con3 primer set used for routine VP4 genotyping (Gentsch et al., 1992). Complete genome sequencing will be performed on representative Rochester G12P[8] strains to characterize these strains at the full genomic level.

5. Conclusion

In conclusion, genetic characterization of G12P[8] strains from a large outbreak associated with severe disease found that the Rochester G12P[8] VP7 and VP4 genes appear to be similar to worldwide human G12-III lineage strains. Selection analyses identified one positively selected site in VP7 and strong purifying selection at multiple sites within VP7

and VP4. The mechanisms behind the evolutionary changes in G12P[8] strains identified in this study, and their ramifications for viral pathogenesis, remain to be determined.

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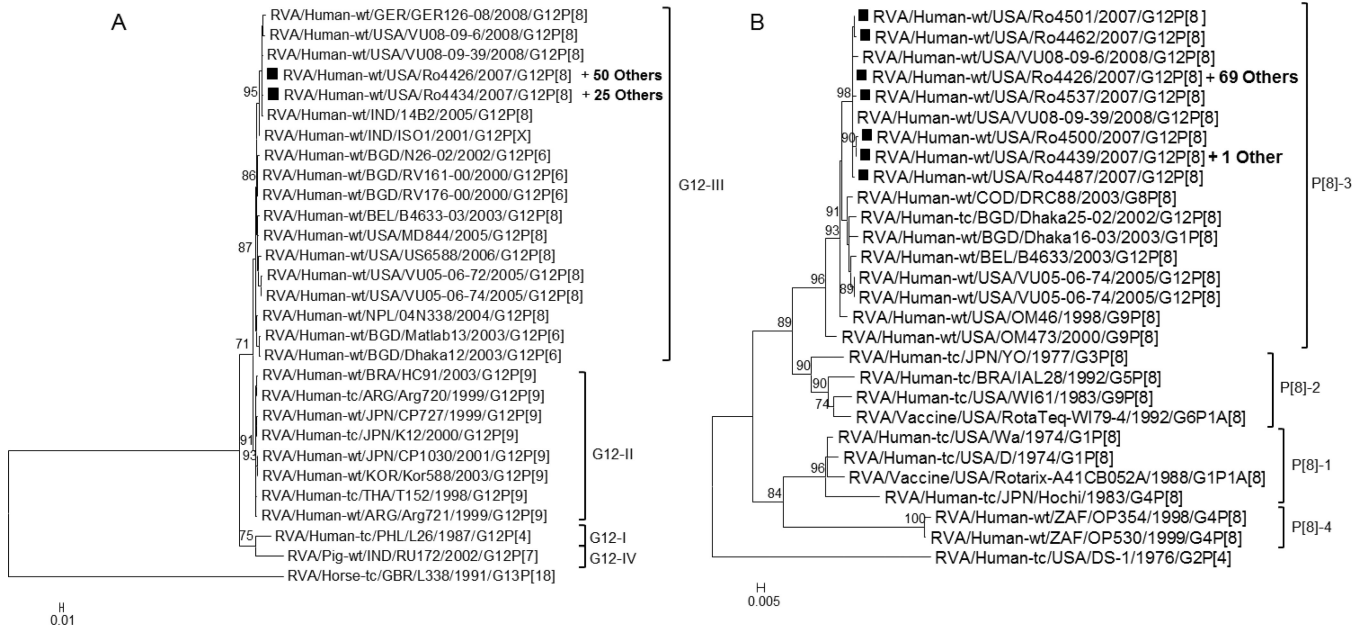


Figure 1. Phylograms indicating genetic relationships of partial VP7 (A) and VP4 (B) nucleotide sequences of G12P[8] strains from Rochester collected during 2006–2007 RVA season (bold text) with representatives of known RVA G12 and P[8] genotypes, respectively. Evolutionary relationships were inferred using PhyML 3.01, and the GTR+I (VP7) and the HKY+G (VP4) models were used to compute evolutionary distances. Numbers next to the nodes are percentages of aLRT statistics computed in SeaView version 4. Only aLRT support greater than 70% percent is shown. Lineages are indicated on the right-hand side. Strain names are presented using the nomenclature proposed by the Rotavirus Classification Working Group (Matthijnsens et al., 2011). Nucleotide sequence of RVA/Human-wt/USA/Ro4532/2007/G12P[8] is identical to that of strain RVA/Human-wt/USA/Ro4439/2007/G12P[8] and thus was omitted from final analysis and has been represented as “+1Other” next to Ro4439.

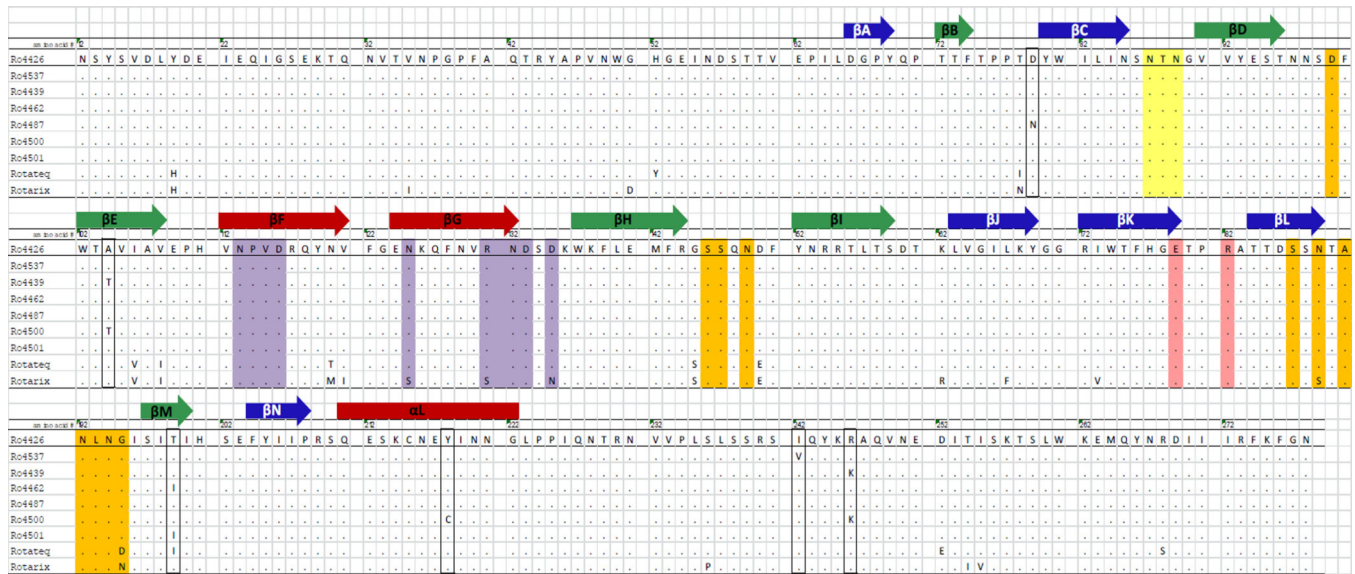


Figure 2.

Amino acid alignment of the VP4 gene for seven Rochester G12P[8] strains (RVA/Human-wt/USA/Ro4426/2007/G12P[8], RVA/Human-wt/USA/Ro4437/2007/G12P[8], RVA/Human-wt/USA/Ro4439/2007/G12P[8], RVA/Human-wt/USA/Ro4462/2007/G12P[8], RVA/Human-wt/USA/Ro4487/2007/G12P[8], RVA/Human-wt/USA/Ro4500/2007/G12P[8], RVA/Human-wt/USA/Ro4501/2007/G12P[8]) and RotaTeq and Rotarix vaccines. Amino acid # indicates position of amino acid. Four VP8*antigenic residues are 8-1 (orange), 8-2 (pink), 8-3 (purple) and 8-4 (yellow) (Zeller et al., 2012). Secondary structure assignments are shown as arrows (β -strand) or rods (α -helix). The colors of the arrows and rods match the colors of the strands and helix in Figure 3. Outline boxes indicate VP4 amino acid changes found in the Rochester G12P[8] strains. Amino acid sequence of RVA/Human-wt/USA/Ro4532/2007/G12P[8] is identical to that of strain RVA/Human-wt/USA/Ro4439/2007/G12P[8] and thus was omitted from the figure.

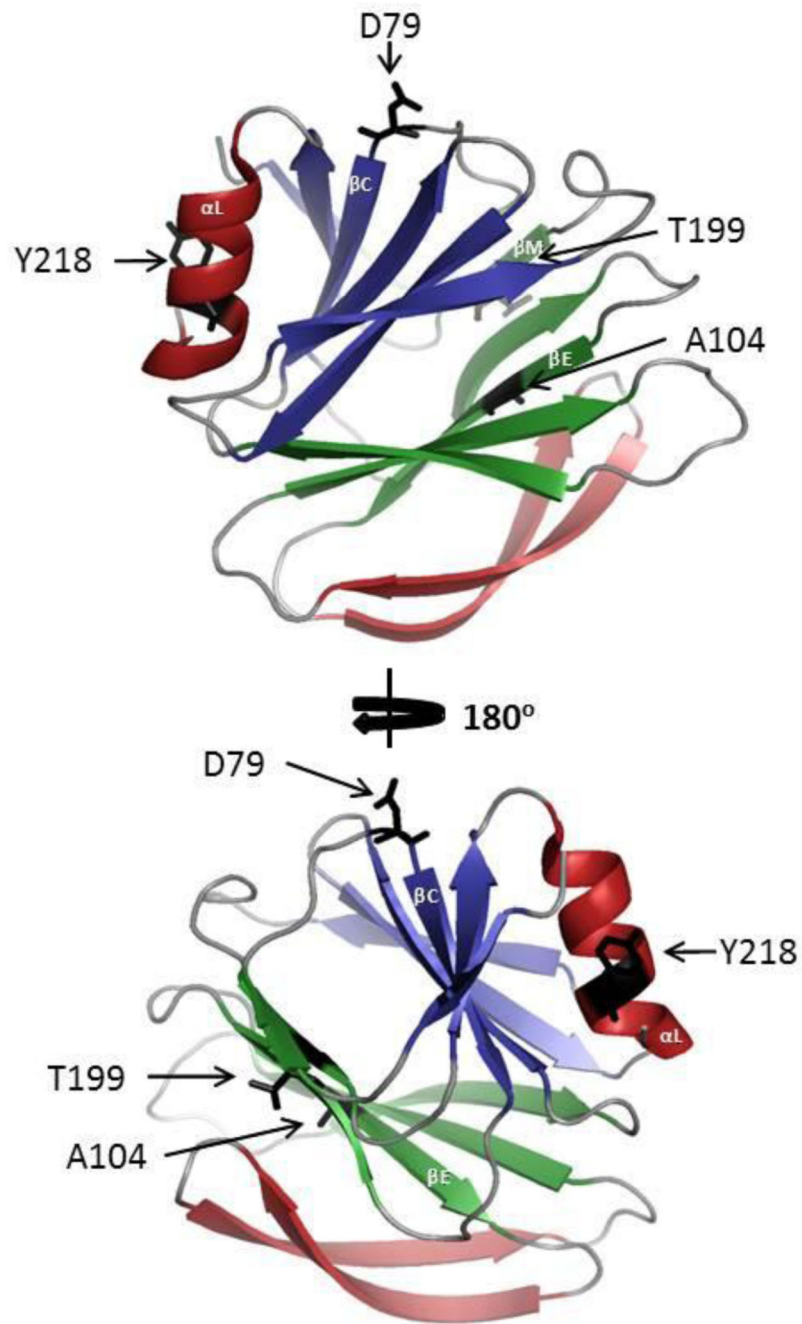


Figure 3. Crystal structure of Wa VP8* (2DWR_A) viewed in MacPymol (<http://www.pymol.org/>). The colors of the secondary structure match the colors of the strands and helix in Figure 2. Labeled amino acids indicate changes found in Rochester G12P[8] strains. The lower structure depicts the upper structure rotated 180° around the vertical axis.

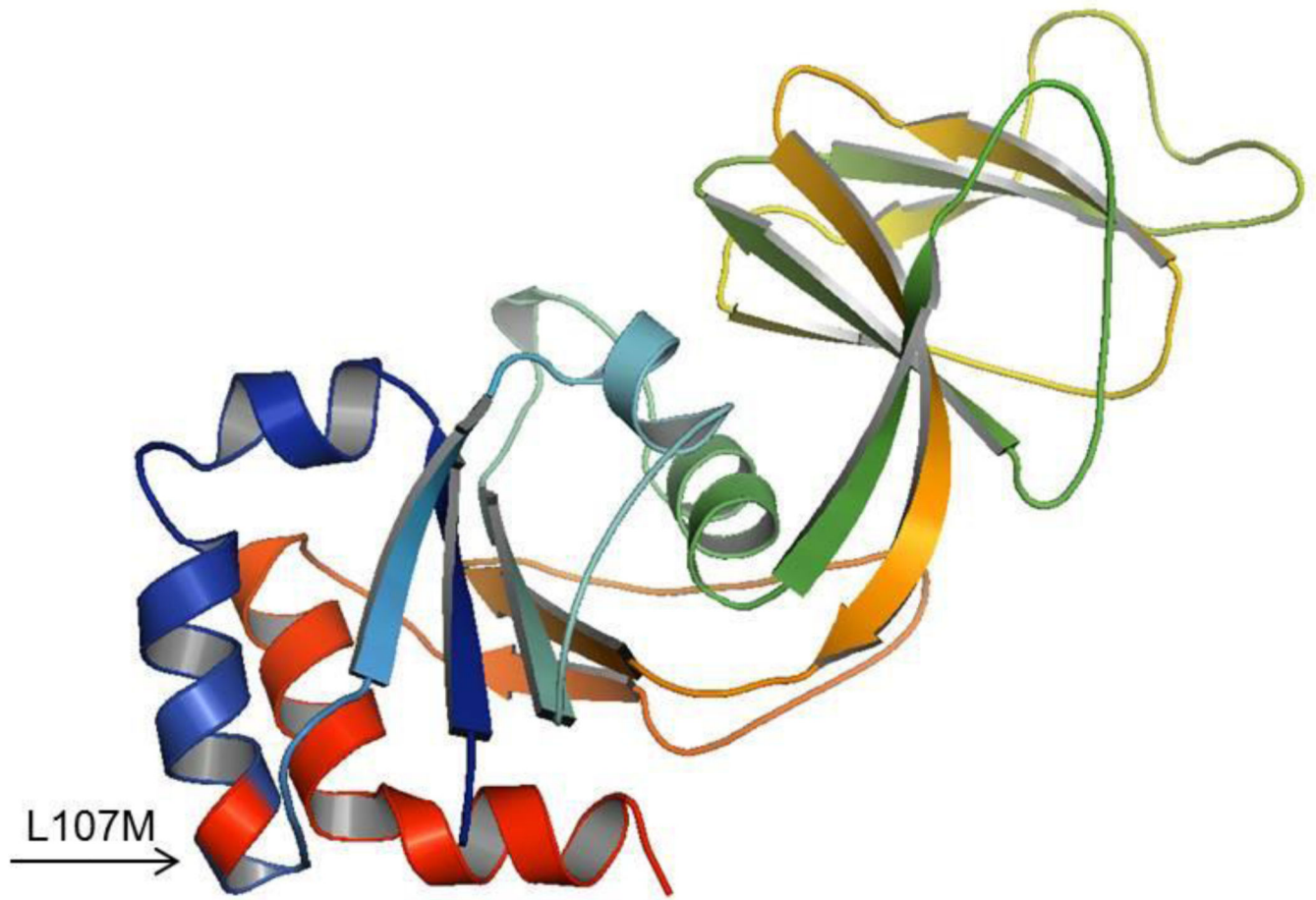


Figure 4. Crystal structure of RRV VP7 (3FMG). Labelled amino acid indicates positively selected site at position 107, which is present in helix B (α B) of the crystal structure.