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Sequence analysis and structural implications of rotavirus capsid proteins

N. PARBHOO, J. B. DEWAR, S. GILDENHUYS*

Department of Life and Consumer Sciences, College of Agriculture and Environmental Sciences, University of South Africa, Private Bag X6, Florida, Roodepoort, South Africa, 1710

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Summary. – Rotavirus is the major cause of severe virus-associated gastroenteritis worldwide in children aged 5 and younger. Many children lose their lives annually due to this infection and the impact is particularly pronounced in developing countries. The mature rotavirus is a non-enveloped triple-layered nucleocapsid containing 11 double stranded RNA segments. Here a global view on the sequence and structure of the three main capsid proteins, VP2, VP6 and VP7 is shown by generating a consensus sequence for each of these rotavirus proteins, for each species obtained from published data of representative rotavirus genotypes from across the world and across species. Degree of conservation between species was represented on homology models for each of the proteins. VP7 shows the highest level of variation with 14–45 amino acids showing conservation of less than 60%. These changes are localised to the outer surface alluding to a possible mechanism in evading the immune system. The middle layer, VP6 shows lower variability with only 14–32 sites having lower than 70% conservation. The inner structural layer made up of VP2 showed the lowest variability with only 1–16 sites having less than 70% conservation across species. The results correlate with each protein's multiple structural roles in the infection cycle. Thus, although the nucleotide sequences vary due to the error-prone nature of replication and lack of proof reading, the corresponding amino acid sequence of VP2, 6 and 7 remain relatively conserved. Benefits of this knowledge about the conservation include the ability to target proteins at sites that cannot undergo mutational changes without influencing viral fitness; as well as possibility to study systems that are highly evolved for structure and function in order to determine how to generate and manipulate such systems for use in various biotechnological applications.

Keywords: rotavirus; capsid protein; amino acid sequence conservation; protein structure; consensus; vaccine candidate

Introduction

Rotavirus infects many species from mammals to birds resulting in gastroenteritis, with group A rotavirus predominantly affecting humans. Rotavirus is the cause of ~450 000 deaths annually worldwide (Parashar *et al*., 2003, 2009; Tate *et al*., 2012) with the highest mortality occurring in the developing world (Tate *et al*., 2012). Rotavirus infection has also been implicated in the onset of type I diabetes in infants (Honeyman *et al*., 2000). Surveillance of circulating rotavirus strains report a continuous change in human strains highlighting the need for continuous characterization of new strains (Collins *et al*., 2015) and occurrence of co-infection, re-assortment as well as interspecies transmission (Jere *et al*., 2001; Papp *et al*., 2014) from porcine to human (Nagai *et al*., 2015; Nyaga *et al*., 2015) or bovine to human (Nyaga *et al*., 2015; Nemoto *et al*., 2015). Nyaga *et al*. (2015) also showed a clear evolutionary relationship between porcine, bovine and ovine rotavirus sequences, indicating that relatively recent interspecies transmission and re-assortment has taken place. Such reports raise concerns as these changes in circulating rotaviruses may have implications on rotavirus vaccine efficacy. The currently administered live rotavirus vaccine, Rotarix® was developed using a strain isolated more than 20 years ago (Ciarlet and Schödel, 2009; Ward and Bern-

*Corresponding author. E-mail: gildes@unisa.ac.za; phone:+27114713295.

Abbreviations: VP = viral protein; PDB = Protein Data Bank

stein, 2009; Plosker, 2010). It has been effective in lowering hospital admissions of diarrhoeal patients (Plosker, 2010; Wang *et al.*, 2010) but its efficacy is lower in the developing world, including Africa and Asia (Molbak *et al.*, 2000; Madhi *et al.*, 2010; Cunliffe *et al.*, 2012; Sow *et al.*, 2012; Zaman *et al.*, 2010). Therefore, solutions need to be sought for future vaccines and treatment strategies. Currently, treatment is limited to symptomatic relief and oral rehydration to prevent dehydration and maintenance of the correct fluid and electrolyte balance. It must be mentioned that there are studies where treatment of the virus with antivirals such as nitazoxanide (Rossignol *et al.*, 2006) and immunoglobulin (Sarker *et al.*, 1998) has been tested and showed to be effective. The treatment with nitazoxanide was based on its ability to inhibit a broad range of viruses (Rossignol *et al.*, 2006) and is, therefore, not a targeted drug designed to act on specific rotavirus targets. More recently, however, novel pyrrole and pyrrolopyrimidine compounds have been synthesized and showed anti-viral activity but have yet to be clinically tested (Mohamed *et al.*, 2015).

Rotavirus belongs to the *Reoviridae* family and its genome comprises 11 segments of double stranded RNA (dsRNA) encoding six structural and six non-structural proteins. The genome is enclosed in a capsid formed by three layers of proteins (Estes and Cohen, 1989). Each dsRNA segment encodes a single protein, apart from gene 11 which encodes two proteins. The inner capsid layer of the virion encapsulates the genome and is mainly comprised of VP2. The trimeric VP6 protein makes up the middle layer of the virion and is used to classify rotavirus subgroups. The outer layer of the virion is made up of VP4, the spike protein that binds to the host cell and, particularly, VP7, the glycoprotein that determines the serotype of rotavirus (Estes and Cohen, 1989). As described by Boyle and Holmes (1986), the structural organization of rotaviruses means that the inner leaflet of the VP2 capsid protein interfaces mainly with viral gene segments while its outer surface associates with the inner surface of the intermediate VP6 capsid layer. The outer surface of this intermediate capsid layer, in turn, associates with the inner surface of the outer capsid, VP7 protein layer. A sequenced-based classification system is now used for rotaviruses (Matthijnssens *et al.*, 2008, 2011).

A plethora of rotavirus sequences have been used in order to identify conserved regions in the rotavirus proteins within and across species. These sites of variability are then analyzed according to their effect on protein structure by overlaying sequence information onto the structure for each protein to determine the role they may play in the molecular function of the virus. Implications are discussed for future drug design, vaccine development and molecular research involving structure and function of the capsid system in infection and biotechnological applications. Although knowledge of genomic sequence data for current and future rotavirus strains is crucial for subtyping and understanding the morphogenesis of the virus, it is equally important to determine to what extent the virus protein products are affected by such genome changes and what the implications may be of such protein variability.

Materials and Methods

Sequence retrieval. Amino acid sequences of human, cow, pig and horse rotavirus VP2, VP6 and VP7 proteins were retrieved from the NCBI database in FASTA format using the search string "VPx AND rotavirus A AND (organism) AND (amino acid full length)[sequence length]".

Consensus sequence deduction. The consensus sequence and conservation score for each protein from each species was determined using CLC Genomics Workbench software. The FASTA format for each species and capsid protein was imported into CLC Genomics Workbench software. The alignment score and consensus sequences were generated using the "create alignment" tool.

Homology model generation and sequence conservation mapping. Homology models of each of the proteins were constructed using the homology builder in the Accelrys Discovery Studio v3.5 suite of tools using PDB codes: 3KZ4 (VP2); 1QHD (VP6) and 3FMG (VP7) as the model templates. An alignment variability score was assigned using ProtSkin (Ritter *et al.*, 2004). Structure renderings according to conservation were performed using PyMOL (The PyMOL Molecular Graphics System, Version 0.99rc6 Schrödinger, LLC).

Results

Rotavirus sequence data indicate changes occurring over time within circulating viruses. We set out to determine the extent to which these sequence changes have impact on the amino acid level of the major capsid proteins constituting the triple layered particle, especially VP2, VP6 and VP7 across all strains of group A rotaviruses. Rather than focusing on a particular species of rotavirus or from a specific region, the dataset encompasses all full-length protein sequences currently available on the NCBI database for human as well as for some livestock (pig, horse and cow) for group A rotaviruses, allowing us to dissect potential effects of interspecies zoonosis and reassortment if they were to occur. Fig. 1 indicates comparative amino acid consensus sequences for the three rotavirus capsid proteins, VP2, VP6 and VP7. The number of data sequences for each capsid protein for each of host organisms is indicated in Table 1. There is no clear evidence that large segments or stretches of amino acids readily interchange as would be expected if reassortment had taken place. It is interesting to note that the amino acid substitutions at the variable regions for each protein

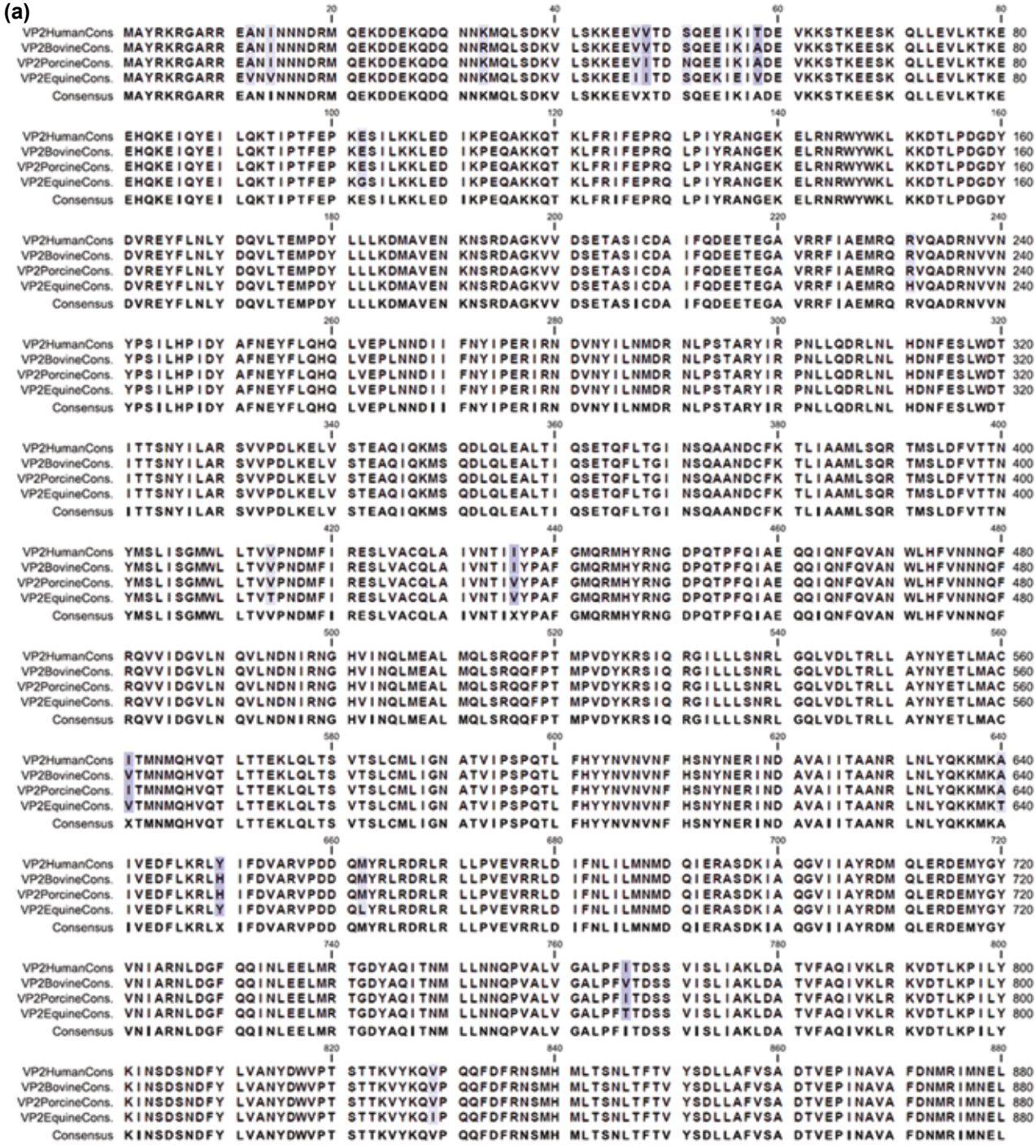

Fig. 1

are conservative, i.e. the same type of residue that usually undergoes substitution and replacement is synonymous, i.e. a polar residue is not replaced by a hydrophobic residue (except in cases where residues are substituted by glycine). Even when examining the sequences chronologically, no distinct pattern of variation can be found.

Structural homology models were then constructed using the homology builder in the Accelrys Discovery Studio 3.5 suite of tools for each of the major capsid proteins using the consensus sequence along with PDB co-ordinates 3KZ4, 1QHD and 3FMG which provided the structure template for VP2, VP6 and VP7, respectively. The homology models

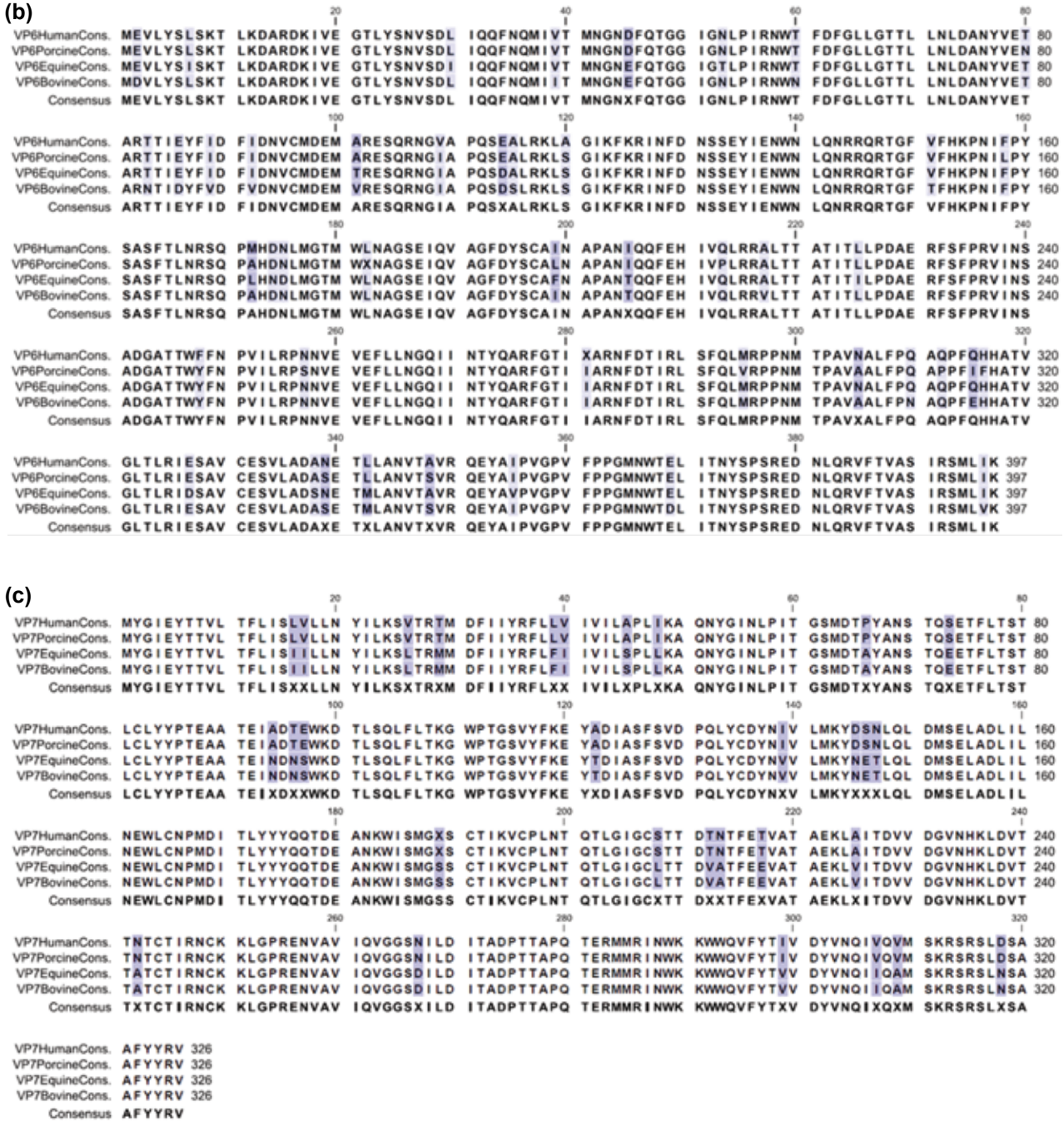

Fig. 1

Deduced consensus sequences

All amino acid sequences for rotavirus proteins VP2 (a) VP6 (b) and VP7 (c) were retrieved from the NCBI database. Amino acids highlighted indicate variation between the consensus sequences. The consensus for each species was computed using the alignment tool of the CLC Genomics workbench suite.

for each of the capsid proteins as a complex with one another are shown in Fig. 2 with the degree of conservation rendered for each protein across species represented by a blue-white spectrum where blue represents variation and white represents no variation (Fig. 3). Numerical data are displayed in Table 2.

Table 1. Number of sequences used for each capsid protein

	Bovine	Equine	Porcine	Human
VP2	78	18	17	114
VP6	166	23	207	540
VP7	327	67	272	570

VP2

The sequence variation of the inner capsid protein, VP2, is low, i.e. 95–97 % of amino acid content has complete conservation within and across species with only 1–17 sites having less than 70% conservation. This correlates with previous findings indicating 84% amino acid similarity (McDonald and Patton, 2008). This inner capsid protein, has few sites of variability on either interface surface (Fig. 3a) owing to the necessary interactions with VP6 on the outside and the viral polymerase (VP1) and the non-specific interactions with the nucleic acid on the inside (Boyle and Holmes, 1986). The core shell domain of VP2 is also said to activate VP1 polymerase activity (McDonald and Patton, 2011), rather than just the N terminus of VP2. The low variability would also mean that subgroup detection using antibodies to VP2 as proposed and tested by McDonald and Patton (2008) is a more viable option than antibodies to VP6 or VP7. The most variable region on the protein is located on the N-terminus (residues 1–81) as has been reported previously by McDonald and Patton, (2011) but could not be mapped as these residues are not modeled in the original PDB file and has not been modeled or any structure determined (Trask *et al.*, 2012). It is a region that makes up the 'fivefold hub' with other VP2 N-termini (McClain *et al.*, 2010) and is responsible for VP1 and VP3 encapsidation (Zeng *et al.*, 1998), RNA interactions and efficient RNA synthesis (McDonald and Patton, 2011). The amino acid content in this region is also polar and positively charged across all the sequences which correspond to the hydration region of the 'fivefold hub' (McClain *et al.*, 2010).

VP6

The middle capsid protein, VP6, also shows relatively high conservation with at least 87% of the amino acid content of the protein being 100% conserved and with 1–32 sites having less than 70% conservation. This latter figure accounts for less than 8% of the protein. The regions of variability

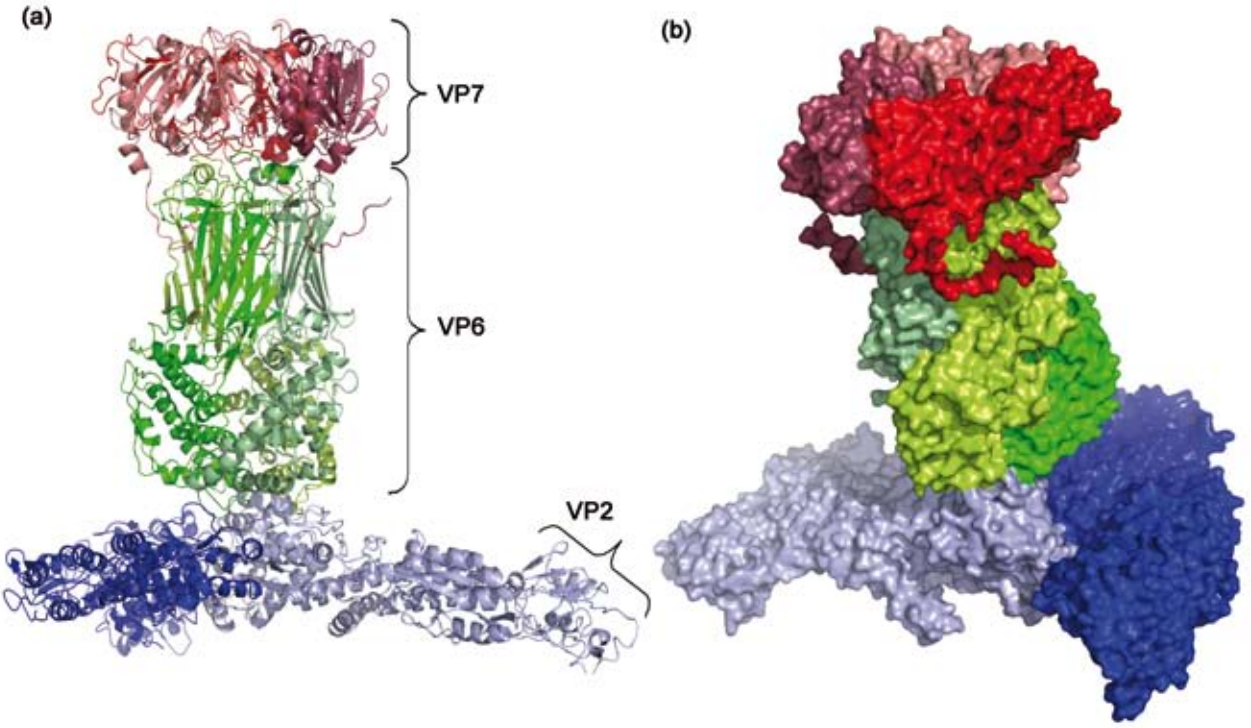

Fig. 2

VP2/6/7 complex

Cartoon representation (a) and surface representation (b) of the complex of the three major capsid proteins in rotavirus. The dimeric complex of VP2 and the trimeric complexes for VP6 and VP7 are shown.

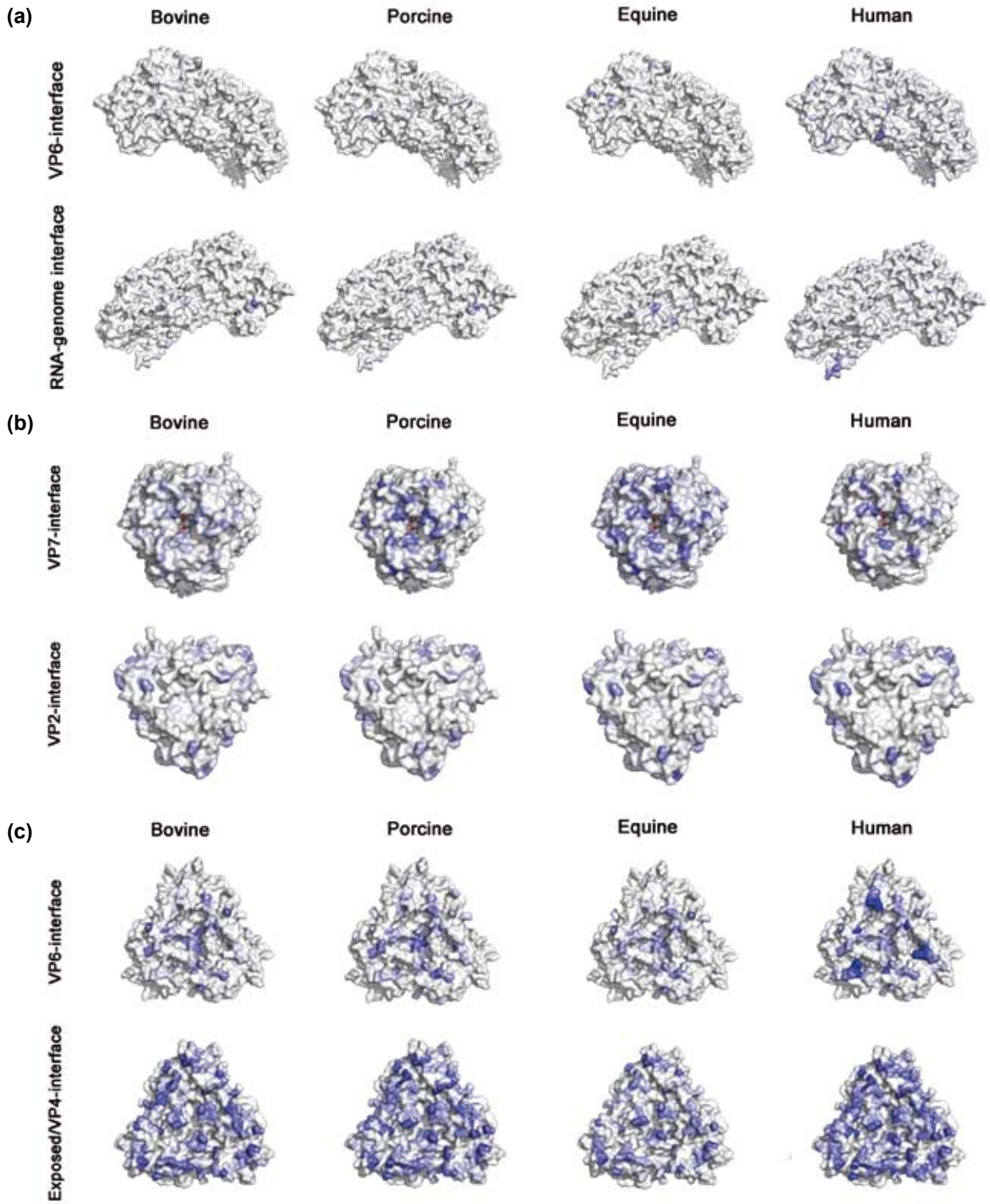

Fig. 3

Structural homology models of the major capsid proteins

Existing PDB co-ordinates were used as the template for generating homology models using the deduced consensus sequence for VP2 [PDB:3KZ4] (a) VP6 [PDB:1QHD] (b) and VP7 [PDB:3FMG] (c). Structures are colored according to the degree of conservation where white represents highly conserved sites and blue represents high variation within each species. Homology models were generated using Accelrys Discovery Studio 3.5. Structure renderings according to conversation were performed using ProtSkin (Ritter *et al.*, 2004) and PyMOL (The PyMOL Molecular Graphics System, Version 0.99rc6 Schrödinger, LLC).

for VP6 are scattered across the protein but overall show a lower degree of variability than VP7 (Fig. 3b). It has been reported that a single mutation at position 172 or 305 could alter subgroup antibody specificity (Löpez *et al.*, 1994) but in Fig. 1b it is evident that there is variation at both of these sites across species. It is, therefore, not surprising that currently there are strains of rotaviruses that can no longer be allocated to one of the two subgroups (Desselberger and Iturriza-Gomara, 2001).

The VP6 protein, being the middle capsid layer protein, interacts with both the outer coat protein, VP7 (residues 271 to 342 (Gilbert *et al.*, 2001) and the inner protein, VP2 (Charpilienne *et al.*, 2002) (Fig. 2). Furthermore, VP6 exists as a trimer in the virion (Mathieu *et al.*, 2001) and thus successful formation of the mature virion is dependent on these interactions making them sterically- and orientation-specific. This can only be achieved through well conserved interactions and hence, may maintain the pressure on amino acid sequences to be well conserved. VP6 is also instrumental in replication of the virus (Boudreaux *et al.*, 2015). On cell entry the loss of VP7 initiates a conformational change in the VP6 trimers (Libersou *et al.*, 2008) "switching" the virus from protecting the genome to inducing the viral polymerase VP1, present in the virus core, to synthesize mRNA. A mutational study of VP2-VP6 interacting residues indicated that the conformational change modifies the VP6 to allow the transcripts to exit (Charpilienne *et al.*, 2002). It has been proposed that protein conformational changes are small rearrangements that are initiated through one of many possible pathways or networks within the proteins (Dokland, 2002). The switch mechanism associated with VP6 during the early stages of infection implies the need for relative conservation of the VP6 sequence, protein or multimer. All these factors indicate that there are many pressures on this protein and therefore, it is not surprising that there is a high level of conservation in the amino acid sequence within and across species.

The VP7 protein of blue tongue virus is almost identical in terms of sequence and structure to VP6 (Charpilienne *et al.*, 2002). This would imply that these proteins have evolved to a current optimal structure that is fit for purpose. This generates an opportunity that we can exploit viral proteins to treat viral infection and therefore, it is not surprising that VP6 has also been proposed as a second generation vaccine candidate (Ward and McNeal, 2010). In a mouse model, neutralizing antibodies to VP6 have been shown to protect against infection by stopping replication (Lappalainen *et al.*, 2014) and a significant reduction in viral shedding was observed in faeces of immunized mice. These results suggest a significant role for mucosal rotavirus VP6-specific IgA for the inhibition of viral replication *in vitro* and *in vivo*. The long term efficacy of vaccines using VP6 protein as the main antigen may be assured due the limited tolerance to changes as seen in sequence conservation (Table 2; Fig. 3b). Reassortment between VP6 and VP7 of rotavirus B (Marthaler *et al.*, 2014) occurs in a fairly random pattern and does not appear to affect large segments of the amino acid sequences or sections of the protein structure.

Table 2. Degree of sequence variation across protein for each species

	Bovine	Equine	Porcine	Human
VP2	3 (27)	5 (44)	5 (40)	5 (41)
VP6	12 (47)	13 (52)	13 (51)	11 (43)
VP7	30 (95)	27 (89)	33 (110)	31 (102)

Variation expressed as a percentage of amino acid content. Actual No. of differing sites is shown in parentheses.

VP7

Regarding VP7, 72% of the amino acid content of this outer capsid protein is always conserved with 14–65 sites having lower than 70% conservation. These latter data account for just 19% of the protein. The epitopes of VP7 identified at regions 87–96 (Green and Kapikian, 1992) correlate with the hypervariable sites on the protein (Fig. 1c). This pattern of variation is also conserved across species (Fig. 3c). The VP7 protein, like VP6, exists as a trimer, forming a co-ordinate complex with Ca^{2+} ions (Dormitzer *et al.*, 2000). However, in contrast to VP6, VP7 has fewer points of contact with neighboring proteins (Fig. 2). The conserved amino acids of VP7 correspond to the regions of subunit interaction of the trimer where negatively charged amino acids containing carboxyl groups are required to co-ordinate around the Ca^{2+} ion as well as residues of the glycosylation site (69 and 71). The glycosylation of VP7 has been said to facilitate the correct disulphide bond formation and folding of rotavirus VP7 (Mirazimi and Svensson, 1998). Disulphide bond rearrangements have been shown in thrombin to allow the protein to undergo an allosteric or conformational switch from an active to inactive form (Huntington and Esmon, 2003). A similar mechanism may function in the VP7 which has 4 disulphide bonds, allowing it to convert from soluble form into the conformation that attaches to VP6 and displaces the lipid layer around the double layered viral particle that then results in the functional triple layered viral particle. Conservation is also seen on the inward facing surface of the VP7 trimer where negatively charged amino acids predominate (Fig. 3c). This is the side of the VP7 protein that interacts with VP6. In contrast, variability exists on the opposite face of the trimer, the face that makes up the exposed surface of the virion. With a selective immune pressure exerted on the outward face, hypervariability in amino acids confers a survival advantage to the virus so that it may escape antibody neutralization.

Discussion

Rotavirus RNA polymerase possesses no proofreading ability and thus we would expect to find a random pattern of nucleotide sequence changes. This is supported by the plethora of sequence data available for rotavirus genomes, which show continuously occurring changes (Matthijnssens and van Ranst, 2012). The nucleotide sequence changes should then translate into a random pattern of amino acid changes across the proteins expressed from the RNA segments. However, the results presented here show that changes are limited to a small number of defined locations on the expressed structural proteins, even across species (Fig. 1 and 3). The proteins must, therefore, have a significant number of amino acids that are essential for structural and functional roles to ensure the integrity of the virus and success in the various stages in rotavirus replication. They each represent an advanced multifunctioning system (Zhang *et al.*, 2014). Regions of variability are concentrated on the surfaces that are at some point in the viral life cycle exposed to the host environment. Hence, it could be said that what the proteins sacrifice in structural integrity they gain in evading host defense mechanism.

The conservation of amino acid sequences is deliberate and any introduced variation must remain below a critical threshold – otherwise it almost always leads to removal of the nascent virus from the virus population. The result is the evolution of compact yet effective infectious entities that have produced stable protein capsid structures. These allow us to identify and pursue amino acid sets within these capsid proteins for use as research models to tease out functional relationships not only in the molecular pathogenesis of these viruses but also to provide us with target areas for treatment strategies and vaccine candidates. Recombinant segments of the viral proteins which align to regions of high conservation to produce powerful long lasting vaccines can be expressed. Such drugs and vaccine components should be designed to take full advantage of the regions on each protein which are constant. As seen for influenza vaccine strategies, conserved viral protein segments act as ideal therapeutic targets (Rahn *et al.*, 2015). As an example of a potential rotavirus therapeutic target, VP6 has been shown to interact with heat shock cognate protein hsc70 and this interaction is said to mediate cell entry (Gualtero *et al.*, 2007). The residues on VP6 that are involved in the interaction with hsc70 are amino acids 280–297 and from our data it is clear that this is a highly conserved region (Fig. 1b). Creating a drug that can bind to VP6 region 280–297 would disable possible cell entry and even assembly of the virus. It would also be highly specific and thus should produce fewer side effects.

Furthermore, produced vaccines containing capsid proteins should have long term efficacy. The idea of possible zoonotic reassortment where rotavirus genotypes normally associated with animal infection may infect humans is not unrealistic based on evidence presented here which illustrates the conservation and symmetry associated with critical proteins. Therefore, one would assume that zoonosis should be taking place more often, producing new more virulent strains which bypass the immune system primed with only limited variability in strain specificity. However, the viral proteins seem to be very similar so even if zoonosis took place it would only affect the human population if the rare sequence variation was present in the zoonotic virus strain and that this virus was able to evade the protective immune response to administered vaccines. This emphasizes the need to not only research those molecular viral features that are critical in rotavirus infection but also to determine host factors that predispose individuals towards rotavirus infection as well as the nature of the immune response following vaccination. The current data also may explain why the vaccines are still relatively effective even if possible zoonosis is taking place because the components of the current vaccines elicit immune responses that may be aimed at epitope regions that are conserved across species.

Nonetheless, assuming that rotavirus capsid proteins are highly evolved, they could be seen as ideal models for the design of drug delivery shells. Mutations in these regions are not represented in the circulating viruses and are, therefore, not tolerated in the capsid system for the full functioning of the virus (Woods, 2015). Some of these functions would be specific to rotavirus but not for production of a basic delivery shell. Full understanding of sequence elements that relate to each functional role will provide insight into how to construct and optimized virus-like particles for use in various applications; aid in diagnostic tools for subtype detection and to design antibodies for non-variable regions on the antigen.

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