Rotavirus VP7 epitope mapping using fragments of VP7 displayed on phages

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Received 5 August 1999; received in revised form 17 September 1999; accepted 1 December 1999

Abstract
cDNA copies of the complete porcine rotavirus CRW-8 VP7 gene were randomly digested to fragments of about 30–60 or 30–500 base pairs by DNase I in the presence of Mn²⁺. The fragments were cloned and expressed in a filamentous phage fd-tet-derived vector to create specific-gene-related peptide libraries. Polyclonal antibodies were then used to pan the SGRP libraries for antibody-binding phages. Analysis of the phage isolates revealed that the majority (86%) of them only had a single insert. However, phages displaying composite inserts containing the VP7 antigenic regions A, B, and C, originally defined by neutralising monoclonal antibody escape mutants, were also isolated. Inserts containing A or C region peptide were found to contain extra sequences from the C region, while the B region epitope was linear and had additional sequence from either upstream or downstream. In addition a dominant and possibly non-neutralising VP7 epitope was identified around amino acids 263–270. One of the recreated antigenic epitopes has also been fused to the outer membrane protein A (OmpA) of Escherichia coli and shown to maintain its antigenicity. The results in this study may have significant implication for recreation of conformational epitopes and vaccine development.

Keywords: Porcine rotavirus VP7; Conformational epitopes; Phage display

1. Introduction
Rotavirus was first discovered by electron microscopy in 1973 and is believed to be one of the most important pathogens responsible for infantile human and animal diarrhoea worldwide [1]. The virus has a genome of 11 segmented RNAs enclosed by three layers of protein capsids. The core layer contains VP1, VP2, and VP3; the inner capsid is made up of the virus group determinant VP6; and the outer capsid is composed of a major glycoprotein VP7 and a relatively minor and protease-sensitive protein VP4. Both VP4 and VP7 can induce neutralising antibodies and are targets for vaccine development.

Studies with monoclonal antibody escape mutants have helped to identify three major neutralising antigenic regions designated A, B, and C in VP7 [2]. The studies also suggested that the antigenic A and C regions were juxtaposed in the virus particle [2,3]. These antigenic epitopes are thought to be conformational because so far no neutralising monoclonal antibodies (mAbs) have recognised VP7 in Western blotting assays, and some mAbs can select mutations in more than one antigenic region. Recently two more neutralising antigenic regions E and F have been proposed [4,5].

Synthetic peptides corresponding to antigenic regions A, B, and C have been tried but have failed to elicit rotavirus-specific neutralising antibody
responses [6,7]. One VP7-derived peptide reported to induce low titres of anti-rotavirus antibodies was from amino acid 240–255 [6]. Another VP7 peptide which was found to prime animals for anti-rotavirus antibody response was located in a region from amino acids 275–295 [8].

Major efforts have been devoted to the development of effective rotavirus vaccines for human and animals since the discovery of the virus [9]. Live attenuated rotaviruses, rotavirus reassortants, virus-like particles made up of rotavirus VP4, VP6, and VP7 proteins, DNA vaccine, and expressed rotavirus outer capsid proteins (VP4 and VP7) in different vectors have all been attempted. A live attenuated rotavirus vaccine consisting of rhesus rotavirus plus reassortants carrying three human G serotype VP7 s (rhesus rotavirus tetravalent or RRV-TV) has recently been licensed in the USA but multiple doses are required [10], making it expensive, and it causes mild side-effects in some populations, so is not yet ideal [11]. Virus-like particles may be a good alternative to live virus in providing protective immunity, but at this stage they are likely to be costly to produce [12,13]. Rotavirus VP7 in recombinant herpes simplex virus is only immunogenic when expressed in cells in presence of other rotavirus proteins [14], whereas VP7 expressed in adenovirus or vaccinia virus [15,16] has been shown to be highly immunogenic although further evaluation of the constructs, particularly the potential side-effect of the virus vectors, has yet to be completed.

VP7 has also been expressed in Dictyostelium discoideum and shown to be both antigenic and immunogenic [17], but further evaluation is needed to assess the practicality of this approach. Recently DNA encoding VP6 has been studied in a mouse model and may offer an alternative vaccine potential [18]. Full and truncated VP7 s have been expressed as chimeric proteins in bacteria, but although some of these fusion products showed antigenicity, they retained poor rotavirus-specific immunogenicity [19]. Since a bacterialvectored vaccine would potentially be much cheaper to produce than the others, but could only succeed if immunogenicity could be improved, we sought a way to reform the conformational epitopes of VP7 so they could be expressed without the framework of the remainder of VP7 and the other adjacent proteins in the rotavirus particle.

The filamentous phage display technique was first described by Smith [20]. There are two major advantages with the technique: it links phenotype and genotype together and it offers the power and convenience to make and screen extremely large libraries. With the developments and improvement of techniques of polymerase chain reaction (PCR) and DNA synthesis in the past few years, phage display has now been widely employed by numerous laboratories in various areas such as protein–ligand interactions, protein engineering, and development of therapeutic agents [21–25].

In this study we use the filamentous phage display method to analyse rotavirus VP7 epitopes and to explore the possibility of reconstituting conformational epitopes for expression in bacterial vaccine vectors.

2. Materials and methods

2.1. Virus and antibodies

Serotype G3 porcine rotavirus CRW-8 and the rabbit anti-CRW-8 serum were obtained from Dr. H. Nagesha [26]. Two pig convalescent sera which contain antibodies against serotype G3 rotaviruses were also characterised by Dr. Nagesha. The anti-CRW-8 mouse ascites was made as described in Ref. [27]. A neutralising monoclonal antibody cocktail was made up of equal volumes of VP7-specific neutralising monoclonal antibodies C1/1, C2/1, C3/1, C2/2, A10, A11, and RV3:5 [28–30]. Each of these mabs has a neutralisation titre >10⁵.

2.2. Phage vectors and bacterial strains

Filamentous phage expression vectors fUSE1, fUSE2, and f88-4 and bacterial strains MC1061, K91, and K802 were kindly provided by Professor George Smith of the University of Missouri. The information on the vectors can be found in earlier publication [31]. Strains for expression of the Omp4::MP31 fusion are E. coli B strain P4188 [32] and E. coli K12 strain C600 [33].

2.3. Phage displayed gene-specific peptide library construction

Porcine rotavirus CRW-8 VP7 gene was reverse transcribed into cDNA and cloned into pUC18. Three silent mutations were introduced into the original CRW-8 VP7 to overcome cloning problems due to a strong E. coli promoter in the sequence [32]. The mutated version of CRW-8 VP7 was designated as CRW-8-VP7m3.

The CRW-8-VP7m3 was excised from the vector, blunt-ended with T4 DNA polymerase and self-ligated using T4 DNA ligase. The ligated VP7-encoding DNA molecules (about 15 μg) were digested down to a size range of about 30–60 b.p or 30–500 b.p (mostly in the 100–180 b.p size range) with DNase1 in the presence of MnCl₂ [34]. The DNA fragments were purified using Chroma Spin 30 (Clontech) and the ragged ends repaired with T4 DNA polymerase (Pharmacia). Excess amounts of DNA fragments (1–2 μg) were used...
in a 200 μl ligation with PvuII-cleaved fUSE1 (about 20 μg) for production of the expression library.

The above ligation was used to transform MC1061 cells by electroporation. A small fraction of the transformed cells was spread onto a Luria–Bertani agar (LB) plate containing tetracycline to determine the transformation efficiency and the size of the libraries. The rest was propagated in LB broth in the presence of tetracycline for 20 h in a shaker incubator at 37°C. Phages were prepared from the culture supernatant by polyethylene glycol precipitation.

2.4. Panning

The phage display gene-specific peptide libraries containing two DNA size ranges were pooled and panned with appropriately diluted antibodies immobilised on petri dishes or microtitre tray wells as described previously [35]. Briefly, about 5 × 10¹¹ to 5 × 10¹² phages were applied to a microtitre well coated with antisera at an appropriate dilution (from 1:50 up to 1:1000 depending on the neutralisation titre) in the first round of selection. Higher dilutions (up to 1:5000) were used for the subsequent second and third rounds in order to isolate higher affinity epitopes. Phages from another phage vector fUSE2, that has an intact pIII and is thus productive, were either mixed with the epitope-displaying phages or used alone as a control. Forty washing cycles were carried out in the third round of panning to remove unbound or loosely bound phage particles. The phages were eluted with 0.1 N glycine solution at pH 2.2.

2.5. Characterisation of the phage isolates

The frequency and the size of the inserts were examined by PCR with a number of randomly picked phage isolates, while the insert origins were determined by sequencing [36].

The effect of the selection was demonstrated by sequence tracking of DNA from cultures prepared from randomly picked phage-derived colonies after different rounds of selection.

The PCR technique was used to screen for potential reconstituted VP7 epitopes. As the starting size of the smallest VP7 fragments was about 30 b.p and as inserts composed of recombined VP7 fragments were desired, phages containing inserts bigger than 50 b.p were further analysed by sequencing.

Single-stranded DNA (ssDNA) was prepared from phages as described previously [34] and sequenced by the dideoxynucleotide chain termination method [36] using a T7 sequencing kit (Pharmacia). Sequence tracking was carried out like sequencing except that only one chain termination reaction was performed.

2.6. Epitope grafting

The VP7 epitopes were initially fused to the phage gene 3-coded protein, pIII, of which the phage has only five copies at one end. In order to increase the copy number of the epitopes and concurrently to test the epitopes in a different background, the inserts were moved from pIII to the phage coat protein pVIII, of which the phage has about 2800 copies. For this purpose another filamentous phage vector f88-4 was used which had an extra copy of pVIII gene inserted for fusion expression. However, the number of pVIII molecules containing foreign peptides may be limited to about 600 copies or less per phage particle, probably due to the interference of these exogenous inserts with the processing of the pVIII pro-coat by leader peptidase at the inner membrane of the E. coli cells [37]. PCR primers containing DNA linkers coding for four glycines or based on the sequences flanking the epitopes were used to transfer the epitopes from pIII to pVIII. The restriction site HindIII or PstI was incorporated into the primers to facilitate directional cloning. The derived phages were analysed by panning for affinity for the relevant antibodies.

2.7. Bacterial expression of OmpA::MP31 fusion

2.7.1. OmpA fusion

One of the selected epitopes, mp31, was also fused to bacterial protein OmpA, under the control of tac or ompA promoter for surface expression in bacteria. We previously demonstrated that part of the porcine rotavirus CRW-8 VP7 spanning amino acids 199–750, could be inserted into the third external loop of OmpA and expressed as a stable chimeric protein associated with the bacterial outer-membrane [32]. Here, the mp31 epitope, flanked by DNA of a KpnI site (included in the PCR primers), was PCR amplified and cloned into the KpnI site of pPR1616 [32] to make plasmid pPR1637. The ompA::mp31 gene is under the control of bacteriophage T7 promoter in pPR1637.

The ompA::mp31 gene was then PCR amplified from plasmid pPR1637 and cloned as a RareI and SalI fragment into the NcoI and SalI sites of plasmid pPR1641 [32] to make plasmid pPR1644. In pPR1644, the ompA::mp31 gene is under the control of the ompA promoter and the distance between the start codon of the ompA::mp31 fusion and the Shine–Dalgarno site of the regulatory region is seven bases compared to nine bases in the wild-type, both in the range for optimal initiation of translation. The ompA::mp31 coding region was also moved into the NcoI and SalI sites of plasmid pPR1662 [32] to make plasmid pPR1664. In plasmid pPR1664, the ompA::mp31 gene is under the control of the tac promoter.
3. Results

A combined primary library of about 109 individual clones was created. Phages were prepared from cultures of the transformed cells after 20 h propagation for subsequent pannings.

Only four phage isolates were selected with the mAb cocktail after the first round of panning and none of these phages contained genuine VP7 protein sequence. Subsequently phage isolates selected by polyclonal antibody were studied.

Three hundred and seventy-six phage isolates were analysed by PCR, and among them, 234 were further studied by sequencing or sequence tracking. All were found to contain inserts and almost all the inserts could be traced back to the original VP7 gene. The sequence tracking results also showed that certain phages containing identical or similar peptides increased over pannings probably suggesting enrichment of the higher affinity epitope-displaying phages (data not shown).

As shown in Table 1, the majority (86%) of these inserts were small single fragments, which went into the vector in different orientations and reading frames (compared to the rotavirus VP7 reading frame) and consequently coded for small peptides (5–15 amino acids) that were frequently “strange” (different from the original VP7 amino acid sequences). These “strange” peptides may have mimicked epitopes of VP7 and other rotavirus proteins or even other non-rotavirus antigens.

Among the bigger inserts (>50 b.p), about 25% were due to fragment recombinations, the others being just single fragments (Table 2), i.e. about 3.5% of the selected phage peptides analysed were due to ligation of multiple VP7 gene fragments. Most of these bigger inserts coded for genuine VP7 peptide sequences. However, no insert larger than 200 b.p was found from phage isolates analysed.

Among the 234 inserts analysed by sequencing, authentic VP7 peptides were mapped over most of the VP7 protein but not evenly distributed (Fig. 1). By far the most frequently isolated phages were those displaying linear VP7 peptides with a common domain located around amino acids 263–270. Among the 152 phage isolates selected with rabbit anti-CRW-8 antiserum, 26 (17%) were identical to RP70 which is located from a.a. 263–283. Other VP7 inserts, although they did not map to the established antigenic regions, may have somehow mimicked them.

The regions in which no phage displayed peptides have been found so far were at amino acids 1–45, 83–86, 102–110, and 226–232. Also worth noting is that no single linear peptide spanning the whole A or C region was found.

The composite epitopes can be highlighted by MP31 and RP30. The MP31 insert was made up of four VP7 DNA fragments which coded for the entire antigenic A region flanked by parts of the C region and other sequences, while the RP30 insert contained three VP7 fragments and encoded two copies and a fragment of the antigenic C region.

Another two isolates, B23 and W41, selected using pig convalescent sera were found to contain a full B region with extra sequence directly from either upstream or downstream. Phages displaying VP7 peptides corresponding to the recently proposed antigenic regions E and F [4,5] were also isolated.

Surprisingly, cross-panning assay showed that none of the bigger epitopes selected appeared to bind to sera other than the one used for their isolation. However, when fused to OmpA, the MP31 epitope, which was initially selected with anti-CRW-8 mouse ascites, was detectable by rabbit anti-CRW-8 serum in a Western blot assay (Fig. 2).

A few selected rotavirus VP7 epitopes (MP21, MP23, MP31, MP54, RP30, RP34, RP70, RP105, and RP111) originally displayed as pIII fusions were moved to the major coat protein (pVIII). During the grafting process it was found that the affinity of some of the epitopes (MP31 and RP30 in particular) for the antibodies used for their isolation was decreased or lost. It is assumed that the conformation of the epitopes was distorted when placed in different backgrounds.

The binding of the epitopes to the Abs used for selection can be restored by either including about 80 amino acids from pIII (about 10 amino acids

<table>
<thead>
<tr>
<th>Antibody for selection</th>
<th>No. of isolates analysed</th>
<th>Insert size &lt; 50 b.p</th>
<th>Insert size &gt; 50 b.p</th>
<th>Composite inserts</th>
<th>VP7 peptide</th>
<th>Non-VP7 peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit antiserum</td>
<td>152</td>
<td>120</td>
<td>32</td>
<td>6</td>
<td>89</td>
<td>63</td>
</tr>
<tr>
<td>Mouse antiserum</td>
<td>62</td>
<td>58</td>
<td>4</td>
<td>4</td>
<td>37</td>
<td>25</td>
</tr>
<tr>
<td>Pig antisera&lt;sup&gt;a&lt;/sup&gt;</td>
<td>158</td>
<td>142</td>
<td>16</td>
<td>3</td>
<td>15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>mAb cocktail</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
</tbody>
</table>

<sup>a</sup> Two pig convalescent sera were used to select phages separately and the isolates were screened by PCR to eliminate the small size inserts.

<sup>b</sup> Only the larger inserts were analysed.
### Table 2
Examples of the epitope peptides isolated

<table>
<thead>
<tr>
<th>Name of the epitope&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Amino acid sequence</th>
<th>Corresponding amino acid positions in VP7</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP21</td>
<td>RTERMMRVNWK + nggttrdkddasav</td>
<td>281–290 + non-VP7</td>
</tr>
<tr>
<td>MP23</td>
<td>REKWWPPL + TERMMR + vnyhlfpsqpv</td>
<td>non-VP7 = 281–286 + non-VP7</td>
</tr>
<tr>
<td>MP31</td>
<td>rtpstsvianfsava + <strong>TEAAIEINDNWK</strong> + QTLGIGCLTT + chhfsnslav</td>
<td>non-VP7 = 87–101 + 201–210 + non-VP7 (A region in <strong>bold</strong>, C region underlined)</td>
</tr>
<tr>
<td>MP54</td>
<td>RVNIPYMPSHNLHVVNSYK YLPPFPV</td>
<td>non-VP7</td>
</tr>
<tr>
<td>RP30</td>
<td>R<strong>G</strong>IGCLTTDTNFEVVA<strong>E</strong>KLA + QTLGIGCLTT + vdrkmkpv</td>
<td>204–225 + 201–210 + non-VP7 (C region in <strong>bold</strong>. Another two identical peptides found)</td>
</tr>
<tr>
<td>RP34</td>
<td>RTERMMRVNWK + nggqslifqpv</td>
<td>281–290 + non-VP7</td>
</tr>
<tr>
<td>RP70</td>
<td>RVGGSDL<strong>I</strong>DTADPTTAPLTERTV</td>
<td>263–283 (another 25 found)</td>
</tr>
<tr>
<td>RP105</td>
<td>RPLL<strong>A</strong>QNYG<strong>I</strong>NL<strong>P</strong>ITG + WPTGSVYFKAY</td>
<td>46–72</td>
</tr>
<tr>
<td>RP111</td>
<td>RNYG<strong>I</strong>NL<strong>P</strong>ITGSM + tilkmkpv</td>
<td>52–64 + non-VP7</td>
</tr>
<tr>
<td>B7</td>
<td>R<strong>Q</strong>QTDEANKW<strong>I</strong>SMGSSCTIKVCP<strong>L</strong>NTQ<strong>L</strong>GIGCLTTDTV</td>
<td>176–212</td>
</tr>
<tr>
<td>B20</td>
<td>RPLL<strong>A</strong>QNYG<strong>I</strong>NL<strong>P</strong>ITG + WPTGSVYFKAY</td>
<td>46–61 + 111–119</td>
</tr>
<tr>
<td>B23</td>
<td>R<strong>V</strong>Y<strong>F</strong>KYTDIAFSVDFPDQ<strong>L</strong>CYCD<strong>N</strong>LVM<strong>M</strong>KYDATLQLDV</td>
<td>116–151 (B region in <strong>bold</strong>)</td>
</tr>
<tr>
<td>B40</td>
<td>REKKW<strong>Q</strong>YF<strong>Y</strong>TIVDVY<strong>W</strong></td>
<td>290–306</td>
</tr>
<tr>
<td>B41</td>
<td>R<strong>A</strong>NKWSMG + <strong>F</strong>ES<strong>D</strong>LRD<strong>I</strong>A<strong>C</strong>T<strong>P</strong>C<strong>R</strong>V<strong>N</strong>W<strong>K</strong>G<strong>S</strong>Y<strong>T</strong>S<strong>W</strong>V</td>
<td>181–189 + non-VP7</td>
</tr>
<tr>
<td>B66</td>
<td>RPLL<strong>A</strong>QNYG<strong>I</strong>NL<strong>P</strong>ITGSM + tilkmkpv</td>
<td>46–82</td>
</tr>
<tr>
<td>W15</td>
<td>RV<strong>N</strong>HKL<strong>S</strong>VT<strong>N</strong>TCTIR<strong>N</strong>CKKL<strong>G</strong>PRE<strong>N</strong>VAIVI<strong>Q</strong>V<strong>G</strong>SDILDV</td>
<td>233–270</td>
</tr>
<tr>
<td>W36</td>
<td>RV<strong>A</strong>IQVGVGS<strong>D</strong>LD<strong>I</strong>DA<strong>P</strong>T<strong>T</strong>A<strong>P</strong>L<strong>T</strong>E<strong>R</strong>V</td>
<td>258–283 (L&lt;sup&gt;*&lt;/sup&gt; is mutation from Q)</td>
</tr>
<tr>
<td>W40</td>
<td>R<strong>K</strong>LS<strong>V</strong>T<strong>N</strong>T<strong>C</strong>T<strong>I</strong>R<strong>N</strong>CKKL<strong>G</strong>PRE<strong>N</strong>VAIVI<strong>Q</strong>V<strong>G</strong>SDILDV</td>
<td>236–283 (L&lt;sup&gt;*&lt;/sup&gt; is mutation from Q)</td>
</tr>
<tr>
<td>W41</td>
<td>R<strong>M</strong>KY<strong>D</strong>ATLQL<strong>D</strong>MS<strong>E</strong>L<strong>A</strong>D<strong>L</strong>I<strong>N</strong>E**W</td>
<td>142–163 (B region in <strong>bold</strong></td>
</tr>
<tr>
<td>W51</td>
<td>RV<strong>N</strong>HKLSVT<strong>N</strong>TCTIR<strong>N</strong>CKKL<strong>G</strong>PRE<strong>N</strong>VAIVI<strong>Q</strong>V<strong>G</strong>SDILDV</td>
<td>233–270</td>
</tr>
<tr>
<td>W56</td>
<td>RIV<strong>D</strong>Y<strong>V</strong>NQIVQAMS<strong>K</strong>RS<strong>L</strong>NSAA<strong>F</strong>Y<strong>Y</strong>R<strong>V + G</strong>V</td>
<td>299–326 + Gly</td>
</tr>
<tr>
<td>W58</td>
<td>RT<strong>I</strong>RN<strong>C</strong>KKL<strong>G</strong>PRE<strong>N</strong>VAIVI<strong>Q</strong>V<strong>G</strong>SDILDV</td>
<td>245–270</td>
</tr>
<tr>
<td>W71</td>
<td>rns<strong>v</strong>i<strong>a</strong>wtslas<strong>a</strong>s<strong>h</strong> + NTCTIR<strong>N</strong>CKKL<strong>G</strong>PRE<strong>N</strong>VAIVI<strong>Q</strong>V<strong>G</strong>SDILDV</td>
<td>non-VP7 sequence + 242–270</td>
</tr>
</tbody>
</table>

<sup>a</sup> Isolates starting with “MP” were selected with mouse ascites; the ones with “RP” were the result of rabbit antiserum; and the ones with “B” or “W” were panned out with pig convalescent sera. The authentic VP7 peptide sequences are in upper case, while the non-VP7 sequences are in lower case. The joints between peptide fragments are indicated with “+”, the corresponding amino acid positions of the peptides in the original VP7 are indicated and the previously described antigenic regions A, B, and C are either in bold or underlined. The frequency peptides isolated more than once is also indicated.
from the N-terminus and 70 from C-terminal of the insert) or by adding four glycines (by PCR) to each end of the epitopes. The results suggested that given the proper folding, the displayed VP7 peptides alone were sufficient for rotavirus-specific antibody recognition.

It must be pointed out that MP31 epitope when expressed as pVIII fusion seemed to be extremely unstable. It is not clear whether it is the size or the folding of the insert that causes this instability.

Bacterial expression of OmpA:MP31 fusion protein under the control of either the tac or OmpA promoter was monitored by Western blotting and a chimeric protein of the expected size (42 kDa) was detected by rabbit anti-rotavirus antibodies (Fig. 2). Preliminary data also suggested that the fusion protein induced a rotavirus-specific antibody response.

4. Discussion

Traditionally, antigenic epitopes are mostly defined through peptide mapping, expression of truncated proteins, mutagenesis or sequence analysis of monoclonal antibody escape mutants [2,38–41]. These approaches in general are time-consuming, costly, limited in precision, and generally restricted to continuous epitopes. Filamentous phage displayed random peptide libraries have been used to decode and refine various antigenic epitopes [42–44]. Recently phage displayed
gene-specific peptide libraries have also been used to delineate antigenic epitopes of bluetongue viruses [45]. However, here we demonstrate the possibility of recreating conformational epitopes with composite peptides derived from the original protein sequence.

The isolation of antigenic region A-related epitope in a composite form only may support the notion that the region is required to interact closely with the C region to be recognised by the antibodies. A stretch of the MP31 amino acid sequence TTSVIANFSAVA that closely resembles TTDTNTFEVEA of the antigenic C region is encoded by a complementary strand sequence from nucleotides 704–747 of VP7. The presence of two cysteines in the C region epitope RP30 may be important for the proper folding and isolation of the epitope. In the original VP7 sequence, the proposed C region spanning a.a. 208–223 is flanked by conserved cysteines at a.a. 191, 196, 207, 244, and 249. These conserved cysteines may exert some conformational constraints on the epitopes. The antigenic region B appears to be linear.

The different antisera appear to vary in their ability to select particular epitopes. The exclusive selection of B region epitopes with the pig convalescent sera may mean that antibodies to the B region are important for recovery. It will be interesting to test the B region epitopes W41 and B23 to see whether they can induce protective immune responses against rotavirus infection.

The most frequent isolation of peptides corresponding to a region between amino acids 233 and 283, with a common domain located at amino acids 263–270, may implicate the region as immunodominant. Recently, a neutralising-mAb escape variant with a change at amino acid 264 has been reported [5], so it is possible that the region could induce a protective immune response. However, this region is relatively conserved among all the group A rotaviruses and thus more likely represents a major non-neutralising epitope since very little cross-neutralisation is observed between different G types. It should also be noted that this region contains most of the disintegrin-like sequence identified by Coulson et al. [46] and that the putative integrin ligands GPR, LDI and LDV occur in several (RP70, W15, W36, W40, W58 and W71) of the epitope peptides listed in Table 2.

The extensive distribution of selected peptides in VP7 may suggest that most portions of the protein can induce antibody responses. The majority of these antibodies are not protective against the virus, probably because the target sites can not be accessed in the intact virus or are not important for virus-cell attachment or initiation of infection.

The successful expression and antibody recognition of MP31::OmpA fusion protein further confirms that the composite epitope is relevant to rotavirus. Preliminary tests with the MP31::OmpA fusion also suggests that it can induce a rotavirus-specific immune response (data not shown). The C region epitope RP30 and another epitope RP70 have also been fused to OmpA but their antigenicity and immunogenicity are yet to be analysed.

Assuming that the DNase I generated DNA fragments are equally represented in the phage display library, the DNA fragments coding for authentic VP7 peptides before the selection should be slightly favoured, (about 18%), due to the presence of stop codons in the other reading frames. The failure to isolate fuse 2 phage and the enrichment of true VP7 peptides to about 65% (90% with the larger fragments) after three rounds of panning suggests that the selection is antibody-specific.

Statistically, the probability for an insert containing n DNA fragments will be \((1/a \times 1/b \times 1/c \ldots) \times (1/2)^a\), where \(a\), \(b\), and \(c\) are the number of molecules of a certain sized DNA (in this study, the \(a\), \(b\), or \(c = \text{size of the VP7 coding region} - \text{size of } a, b, \text{or } c\) fragment). For RP30 which is made up of three different VP7 fragments of 66, 32, and 25 b.p respectively, the probability is about \(1.5 \times 10^{-10}\) and similarly, MP31 should occur at a probability of about \(8 \times 10^{-14}\). However, our library size of about \(10^9\) phages indicates that many desirable composite inserts are not represented and this may explain why no relevant phages were pulled out using monoclonal antibodies.

It may be worthwhile to synthesise an MP31-like peptide for some other rotavirus serotypes to see if the epitope will be similarly recognised by the corresponding antibodies. It will be equally important to apply the phage display technique to another serotype of rotavirus to see whether similar recreated VP7 epitopes can be isolated.

**Acknowledgements**

This work was supported by a GIRD grant from the Department of Industry, Science and Technology, Commonwealth of Australia, Cyanamid Australia Ltd and Bioproperties (Australia) Pty Ltd.

**References**


