

Appendix A

A.1 Material transfer agreement (MTA) form between North-West University and University of Limpopo

Diarrhoeal Pathogens Research Unit (DPRU)

A Division of the Department of Virology, University of Limpopo Medunsa Campus



Material Transfer Agreement

This is a legally binding document governing conditions for the transfer of biological material, hereinafter referred to as the “MATERIAL,” and any information relating thereto, hereinafter referred to as the “INFORMATION,” from the DPRU to the Requesting Party, hereinafter referred to as the “RECIPIENT.”

A. RECIPIENT Information

| | | | |
|---|----------------------------|---|-------------------------|
| <i>Name of Requesting Party</i> | North-West University | | |
| Delivery Address (complete street address) | | | |
| Department Biochemistry | Building F3 | Room Number 218 | |
| Street Address 11 Hoffmann Street 11, 2531 Potchefstroom, South Africa | | | |
| City Potchefstroom | Province North-West | Postal Code 2520 | Country South Africa |
| Phone number 018 299 2317 | Fax number 018 299 2363 | e-mail Address Albie.VanDijk@nwu.ac.za | |

B. Researcher(s)/End User(s) Information (Please provide CV(s) of all End User(s))

| First and Last Name | Telephone | Fax | e-mail address |
|---------------------|--------------|--------------|-------------------------|
| Prof AA Van Dijk | 018 299 2317 | 018 299 2363 | Albie.VanDijk@nwu.ac.za |

| | | | |
|-------------------------------|--------------|--------------|------------------------|
| Dr T O'Neill | 018 299 2069 | 018 299 2363 | Trudi.ONeill@nwu.ac.za |
| Khuzwayo C Jere | 018 299 2069 | 018 299 2363 | khuzwayojere@yahoo.com |
| Potential future PhD students | 018 299 2069 | 018 299 2363 | N/A |

Insert more rows as required

C. MATERIAL Information


| |
|--|
| <i>Nature of the MATERIAL Requested</i> |
| Raw stool samples with their previously characterized data, for instance, electropherotypes, genotypes and sequences. No clinical or patient information will be provided. |
| Reason for Requesting the MATERIAL |
| <p>Rotaviruses are the major aetiological agents of diarrhoea and are associated with over 500 000 childhood mortalities annually across the globe (Parashar, <i>et al.</i> 2006). Despite comparable rotavirus incidents reported in both developed and developing countries, majority of rotavirus associated deaths are reported in developing countries (Santos and Hoshino, 2005).</p> <p>Rotavirus vaccines have been developed and licensed in different countries for routine immunization, although protection against natural rotavirus infection can not be achieved. (Vesikari, <i>et al.</i> 2004; Ruiz-Palacios, <i>et al.</i> 2006; Vesikari, <i>et al.</i> 2006). In addition, some differences in the circulating rotavirus genotypes in developed and developing countries, and also emerging strains have been reported (Santos and Hoshino, 2005). This could be a potential set-back towards rotavirus vaccines that have demonstrated good efficacy in developed and Latin American communities (Glass, <i>et al.</i> 2006). The prevalence of G2, G8 and G9 across Africa poses concerns as the vaccines seems to less protect against G2 rotaviruses and also the vaccine formulation did not include G8 and G9 strains (Santos and Hoshino, 2005; Ruiz-Palacios, <i>et al.</i> 2006). Therefore, vaccine development efforts with the main emphasis on strains prevailing in Africa will help in reducing rotavirus related mortalities in Africa (Breese <i>et al.</i> 1999)..</p> <p>Rotavirus-like particles, with the three concentric protein layers but devoid of the genomic material, have achieved induction of both serum and faecal immunoglobulin through intranasal and intramuscular administration in animals (Conner, <i>et al.</i> 1993). In addition, both homologous and heterologous protection against natural infection has been reported (Crawford, <i>et al.</i> 1999). This makes VLPs a potential future strategy for the development of rotavirus vaccines as they are safe to manufacture.</p> <p>Therefore, the requested stool samples will be used:</p> <ol style="list-style-type: none"> 1. dsRNA will be isolated from the stools, amplified as cDNA which will subsequently be sequenced to determine the consensus sequence of the 11 gene segments of the rotaviruses 2. Rotaviruses in the stools will be adapted and isolated from tissue culture to be used in comparative studies 3. The tissue culture adapted rotaviruses will again be molecularly characterised and |

compared to the wild type sequence

4. The consensus sequence obtained for the wild type viruses will be used to construct chimaeric VLPs

5. The consensus sequence obtained for the wild type viruses will be used to engineer recombinant reassortant rotaviruses through reverse genetics.

D. Laboratory Facilities

| | | | | | | | |
|--|-------------------------|--|--|---|---|---|---|
| Laboratory Facilities Available for Receipt, Use and Storage of the MATERIAL | | | | | | | |
| <p>The Division of Biochemistry has a P2 laboratory where all the work will be conducted. The team comprises of post-graduate students, qualified laboratory personnel and qualified supervisors who act as mentors. Each member is well trained to follow good laboratory practices.</p> <p>A -20⁰C chest freezer has been dedicated to the storage of the stool samples. This is an attempt to avoid cross contamination, as much of the work in the department involves tissue culture and molecular biology. In addition, the department has -20⁰C walking freezers that may act as back-up in cases of unexpected events that might affect the performance of the -20⁰C chest freezer.</p> | | | | | | | |
| Biosafety Containment Level of Facility | | | | 1 | 2 | X | 3 |
| Bio-Safety Officer: | Alberdina Aike van Dijk | |  | | | | |
| | First and Last Name | | | | | | |
| | | | Signature | | | | |

E. Conditions of Transfer of the MATERIAL

The MATERIAL and INFORMATION are provided on the following conditions:

1. The RECIPIENT will not permit the MATERIAL and/or the INFORMATION, or any part thereof, to come into the possession or control of any other entity or person, except those who are engaged in the abovementioned research, development, testing and/or evaluation, under the supervision of the RECIPIENT, and who have accepted the same obligations in respect of the MATERIAL and the INFORMATION as set forth in this document.
2. The RECIPIENT will use the MATERIAL and the INFORMATION exclusively for the purpose of the research, development, testing and/or evaluation, described under Section C above. Without the prior written authorisation of the DPRU, the RECIPIENT will not sell, or furnish such MATERIAL and/or INFORMATION to any third party. Except as explicitly provided in this Material Transfer Agreement (including Section C above), the RECIPIENT will not, furthermore, use such MATERIAL and/or INFORMATION in any

- way for the commercial production or sale of any products, or otherwise for commercial purposes.
3. Nothing contained in this Agreement shall restrict the DPRU's right to sell, transfer, assign or distribute the MATERIAL and INFORMATION to any other person for commercial or non-commercial purposes.
 4. Other than explicitly provided herein, this Material Transfer Agreement will not be construed as conveying to the RECIPIENT any rights or title to the MATERIAL and/or the INFORMATION. The RECIPIENT will treat the MATERIAL and INFORMATION as strictly confidential and proprietary to the DPRU, and/or persons or entities collaborating with the DPRU, and will disclose such MATERIAL and INFORMATION only under the same obligations of confidentiality and restrictions on use as those contained herein.
 5. The DPRU will remove all identifiers from the MATERIAL and INFORMATION that might allow it to be traced back to any individual. A minimum amount of clinical and microbiological information will be recorded by the DPRU. No information considered confidential by the DPRU shall be passed along to the RECIPIENT.
 6. Obligations of confidentiality will not apply to INFORMATION which the RECIPIENT can show was in the public domain at the time of its acquisition hereunder, or becomes part of the public domain otherwise than by breach of the undertakings set forth in this Material Transfer Agreement.
 7. The MATERIAL is not appropriate, nor intended, for use in humans.
 8. Any MATERIAL delivered pursuant to this Agreement is understood to be experimental in nature and may have hazardous properties.
 9. The DPRU and persons and entities collaborating with the DPRU make no warranty of merchantability or fitness of the MATERIAL or the INFORMATION for any particular purpose or any other warranty, either expressed or implied, or that the use of the MATERIAL will not infringe any patent, copyright, trademark, or other proprietary rights.
 10. The RECIPIENT agrees that the DPRU has no control over the use that is made of the MATERIAL and the INFORMATION by the RECIPIENT. Consequently, the RECIPIENT agrees that the DPRU shall not be liable for such use. Thus, the RECIPIENT agrees to assume full responsibility for, and to hold the DPRU harmless from, any and all claims and liabilities by third parties resulting from, or otherwise related to, the use, storage or disposal of the MATERIAL, and possession and use of the INFORMATION, as well as of materials incorporating the MATERIAL.
 11. The RECIPIENT will ensure that the MATERIAL will at all times be used and handled in compliance with all relevant laws, rules and regulations applicable to the use of infectious substances and other biological materials.
 12. On completion of the research, development, testing and/or evaluation, using the MATERIAL, the RECIPIENT will cease to use any remaining quantities of the MATERIAL and the INFORMATION for any purpose and, at the direction of the DPRU, either destroy, or return to the DPRU, all such remaining quantities of the MATERIAL and any and all copies of the INFORMATION.

13. Completion of the research, development, testing and/or evaluation, using the MATERIAL, will not relieve the RECIPIENT of any obligations under this Material Transfer Agreement, which, by their nature, are clearly intended to survive termination.
14. Any dispute relating to the interpretation of application of this Agreement will, unless amicably settled, be subject to conciliation. In the event of failure of the latter, the dispute will be settled by arbitration.
15. The arbitration will be conducted in accordance with the modalities to be agreed upon by the parties or, in the absence of agreement, with the rules of arbitration of the International Chamber of Commerce. The parties will accept the arbitral award as final.
16. Nothing in this Agreement shall be interpreted as establishing a partnership between the parties or establishing one party as the agent of the other or conferring a right on one party to bind the other, except as may be specifically set out herein.

This agreement sets forth the entire understanding between the parties and supersedes any prior agreements, written or verbal. It shall only be capable of change by written amendment executed by duly authorised officers of the parties.

I hereby certify that I have read and understood the conditions outlined in this Agreement and I agree to abide by them in the receipt and use of the MATERIAL

Recipient/Scientist: _____H.G. O'Neill_____

Recipient's Organization: ___North-West University_____

Address: ___Biochemisty, NWU, Potchefstroom Campus_____



_____21/05/2009_____

Signature of Recipient/Scientist

Date

I hereby warrant that I, as the Responsible Administrative Authority of the Receiving Party, have the full authority to execute this Agreement and to thereby bind the Receiving Party

Name of Authorized Official: _____

Signature of Authorized Official

Date

Approval by the DPRU:

Details of DPRU Member from whom the MATERIAL will be obtained:

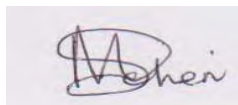
Name: Dr Mapaseka Seheri

Unit/group/Centre: MRC / UL Diarrhoeal Pathogens Research Unit

Address: Department of Virology, School of Pathology & Pre-Clinical Sciences
University of Limpopo, P O Box 173, Medunsa campus, 0204, Pretoria

I hereby warrant that I, as an Authorized Official of the DPRU, have the full authority to execute this Agreement on behalf of the DPRU, and hereby certify my approval of the transfer of the MATERIAL to the RECIPIENT

Name of Authorized Official: Mapaseka Seheri



Signature of Authorized Official

23/07/2009

Date

The relevant signatories must sign each of two copies of this letter, one of which must be retained by the DPRU Member from whom the MATERIAL will be obtained, and one retained by the RECIPIENT.

A.2 Material transfer agreement (MTA) form between North-West University and National Institute for communicable diseases

National Institute for Communicable Diseases (NICD)

A Division of the National Health Laboratory Services



Material Transfer Agreement

This is a legally binding document governing conditions for the transfer of biological material, hereinafter referred to as the “MATERIAL,” and any information relating thereto, hereinafter referred to as the “INFORMATION,” from the NICD to the Requesting Party, hereinafter referred to as the “RECIPIENT.”

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| City Potchefstroom | Province North-West | Postal Code 2520 | Country South Africa |
| Phone number 018 299 2317 | Fax number 018 299 2363 | e-mail Address Albie.VanDijk@nwu.ac.za | |

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| First and Last Name | Telephone | Fax | e-mail address |
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| Dr T O'Neill | 018 299 2069 | 018 299 2363 | Trudi.ONeill@nwu.ac.za |
| Khuzwayo C Jere | 018 299 2069 | 018 299 2363 | khuzwayojere@yahoo.com |
| Potential future PhD students | 018 299 2069 | 018 299 2363 | N/A |

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C. MATERIAL Information

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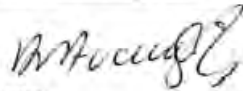
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Laboratory Facilities Available for Receipt, Use and Storage of the MATERIAL

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| | | | |
|--|---|---|---------------------------------------|
| Biosafety Containment Level of Facility | 1 | 2 | <input checked="" type="checkbox"/> 3 |
|--|---|---|---------------------------------------|

| | | |
|----------------------------|--|---|
| Bio-Safety Officer: | ALBERDINA AIKE VAN DIJK First and Last Name |  Signature |
|----------------------------|--|---|

E. Conditions of Transfer of the MATERIAL

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2. The RECIPIENT will use the MATERIAL and the INFORMATION exclusively for the purpose of the research, development, testing and/or evaluation, described under Section C above. Without the prior written authorisation of the NICD, the RECIPIENT will not sell, or furnish such MATERIAL and/or INFORMATION to any third party. Except as explicitly provided in this Material Transfer Agreement (including Section C above), the RECIPIENT will not, furthermore, use such MATERIAL and/or INFORMATION in any way for the commercial production or sale of any products, or otherwise for commercial purposes.
3. Nothing contained in this Agreement shall restrict the NICD's right to sell, transfer, assign or distribute the MATERIAL and INFORMATION to any other person for commercial or non-commercial purposes.
4. Other than explicitly provided herein, this Material Transfer Agreement will not be construed as conveying to the RECIPIENT any rights or title to the MATERIAL and/or the INFORMATION. The RECIPIENT will treat the MATERIAL and INFORMATION as strictly confidential and proprietary to the NICD, and/or persons or entities collaborating with the NICD, and will disclose such MATERIAL and INFORMATION only under the same obligations of confidentiality and restrictions on use as those contained herein.
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9. The NICD and persons and entities collaborating with the NICD make no warranty of merchantability or fitness of the MATERIAL or the INFORMATION for any particular purpose or any other warranty, either expressed or implied, or that the use of the MATERIAL will not infringe any patent, copyright, trademark, or other proprietary rights.
10. The RECIPIENT agrees that the NICD has no control over the use that is made of the MATERIAL and the INFORMATION by the RECIPIENT. Consequently, the RECIPIENT agrees that the NICD shall not be liable for such use. Thus, the RECIPIENT agrees to

assume full responsibility for, and to hold the NICD harmless from, any and all claims and liabilities by third parties resulting from, or otherwise related to, the use, storage or disposal of the MATERIAL, and possession and use of the INFORMATION, as well as of materials incorporating the MATERIAL.

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This agreement sets forth the entire understanding between the parties and supersedes any prior agreements, written or verbal. It shall only be capable of change by written amendment executed by duly authorised officers of the parties.

I hereby certify that I have read and understood the conditions outlined in this Agreement and I agree to abide by them in the receipt and use of the MATERIAL

Recipient/Scientist: H. G. O'NEILL

Recipient's Organization: NORTH-WEST UNIVERSITY

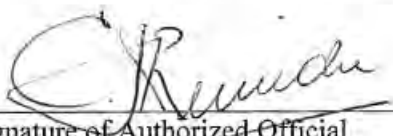
Address: BIOCHEMISTRY, NWU, POTCHEFSTROOM CAMPUS

H. G. O'Neill
Signature of Recipient/Scientist

21/05/2009
Date

I hereby warrant that I, as the Responsible Administrative Authority of the Receiving Party, have the full authority to execute this Agreement and to thereby bind the Receiving Party

Name of Authorized Official: PROF. C. J. REINECKE


Signature of Authorized Official

10-07-2009
Date

Approval by the NICD:

Details of NICD Member from whom the MATERIAL will be obtained:


Name: NICOLA PAGE

Unit/group/Centre: VIRAL GASTROENTERITIS UNIT

Address: ROOM C10, 1 MOOREFURNER ROAD, SANDRINGHAM, 2131

I hereby warrant that I, as an Authorized Official of the NICD, have the full authority to execute this Agreement on behalf of the NICD, and hereby certify my approval of the transfer of the MATERIAL to the RECIPIENT

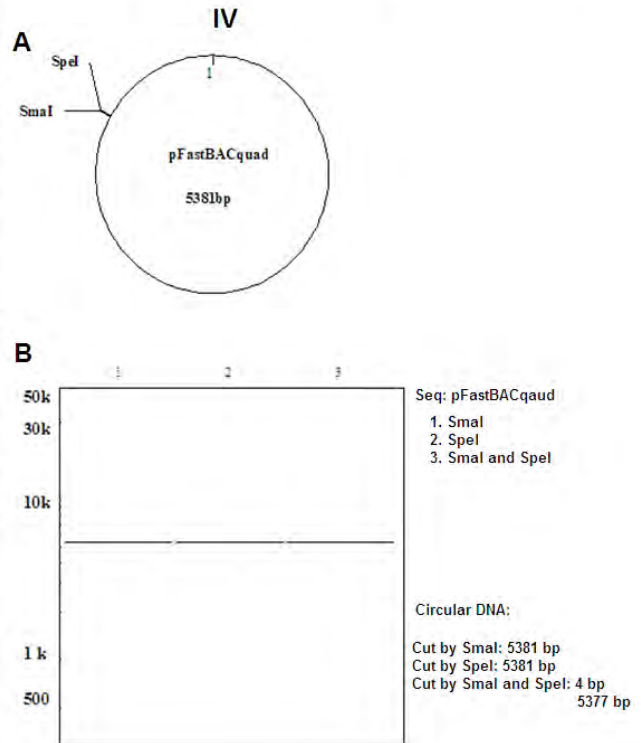
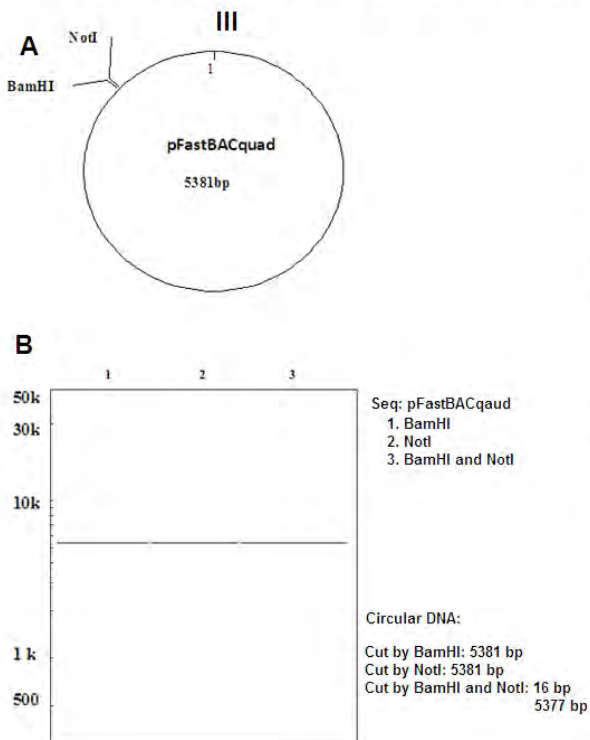
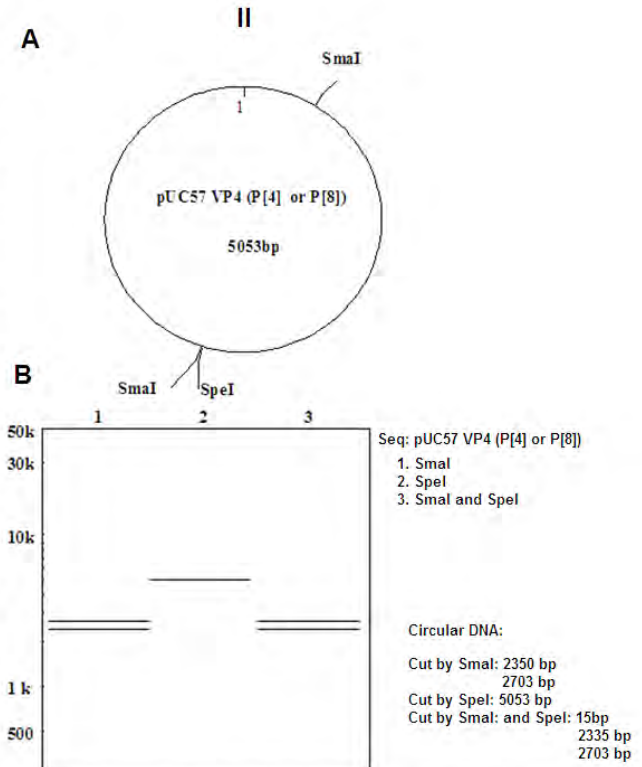
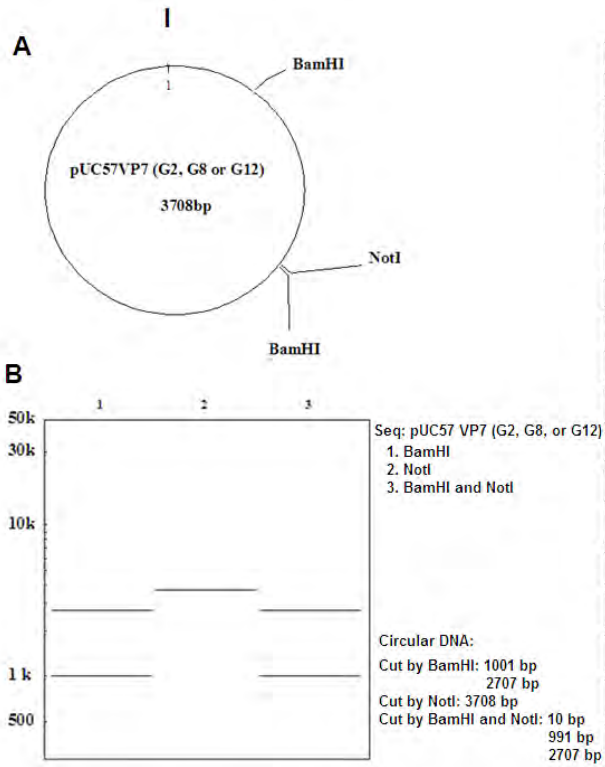
Name of Authorized Official: NICOLA PAGE


Signature of Authorized Official

23-07-2009
Date

The relevant signatories must sign each of two copies of this letter, one of which must be retained by the NICD Member from whom the MATERIAL will be obtained, and one retained by the RECIPIENT.

Appendix B



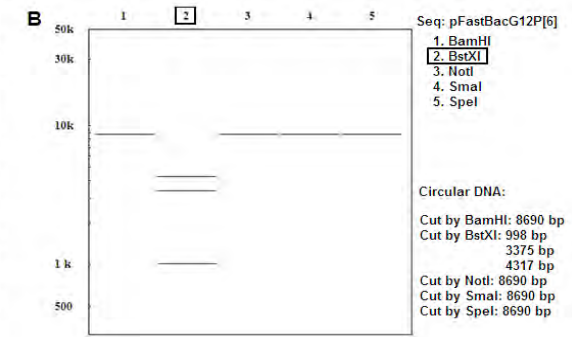
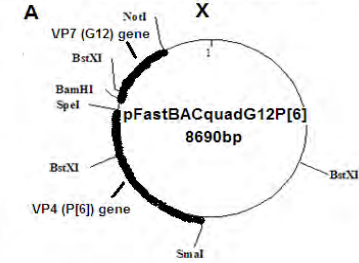
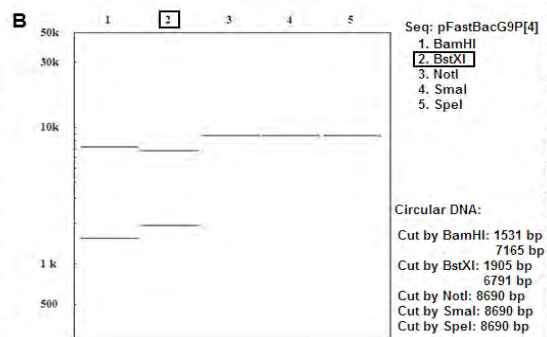
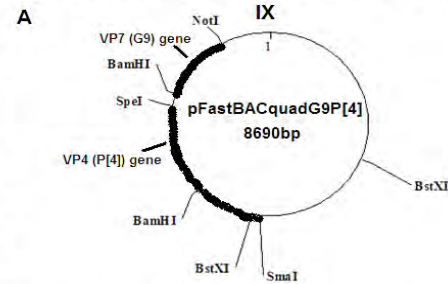
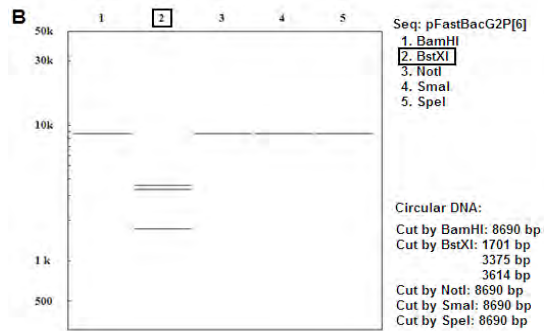
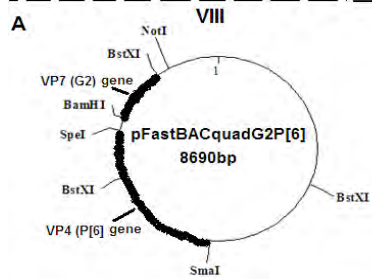
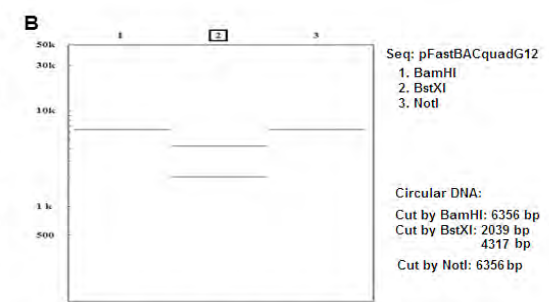
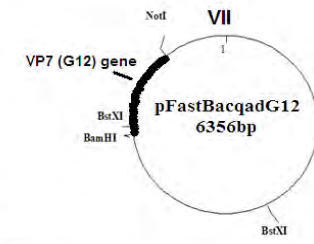
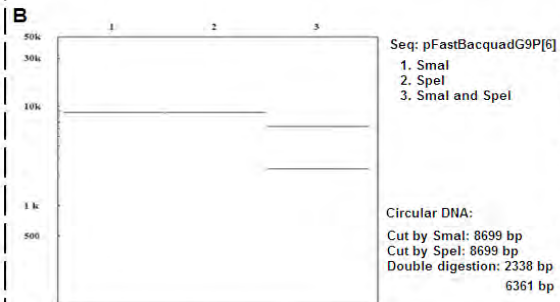
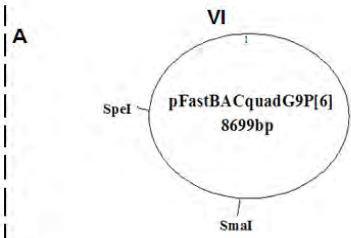
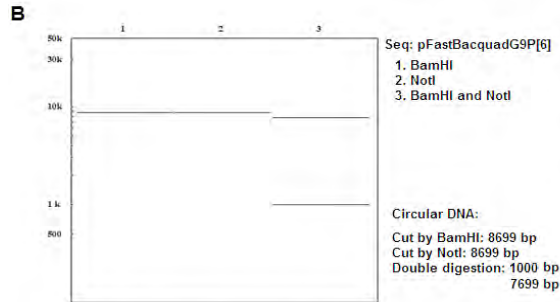
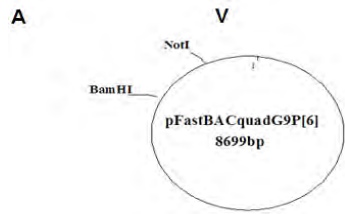


Fig. 1. *In silico* plasmid maps of expected restriction patterns after subjectin various pFBq constructs to various REs. **I)** Restriction map and expected sizes of the fragments of pUC57 VP7 (G2, G8 or G12) plasmid digested with *BamHI* and *NotI*. **II)** Restriction map and expected sizes of the fragments of pUC57 VP4 (P[4] or P[8]) plasmid digested with *SmaI* and *SpeI*. **III)** Restriction map and expected sizes of the fragments of pFBq plasmid digested with *BamHI* and *NotI*. **IV)** Restriction map and expected sizes of the fragments of pFBq plasmid digested with *SmaI* and *SpeI*. **V)** Restriction map and expected sizes of the fragments of the recombinant pFBqG9P[6] expression plasmid digested with *BamHI* and *NotI*. **VI)** Restriction map and expected sizes of the fragments of the recombinant pFBqG9P[6] expression plasmid digested with *SmaI* and *SpeI*. **VII)** Restriction map and expected sizes of the fragments of the recombinant pFBqG12 expression plasmid digested with *BstXI*. The position where the gene segment encoding VP7 protein was cloned is highlighted in black. **VIII)** Restriction map and expected sizes of the fragments of the recombinant pFBqG2P[6] expression plasmid digested with *BstXI*. The position where the gene segment encoding VP4 and VP7 protein were cloned are highlighted in black. **IX)** Restriction map and expected sizes of the fragments of the recombinant pFBqG9P[4] expression plasmid digested with *BstXI*. The position where the gene segment encoding VP4 and VP7 protein were cloned are highlighted in black. **X)** Restriction map and expected sizes of the fragments of the recombinant pFBqG12P[6] expression plasmid digested with *BstXI*. The position where the gene segment encoding VP4 and VP7 protein were cloned are highlighted in black. From I to X, A, restriction map; B, restriction patterns of the plasmid DNA fragments expected on agarose gel. Lane 1; O'GeneRuler DNA Ladder Mix molecular marker (Fermentas Inc., Maryland, USA).

Appendix C

MLERA, L., **JERE, K. C.**, VAN DIJK, A. A. & O'NEILL, H. G. 2011. Determination of the whole-genome consensus sequence of the prototype DS-1 rotavirus using sequence-independent genome amplification and 454® pyrosequencing. *J Virol Methods*, 175, 266-71.

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Short communication

Determination of the whole-genome consensus sequence of the prototype DS-1 rotavirus using sequence-independent genome amplification and 454[®] pyrosequencing

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ABSTRACT

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The prototype DS-1 rotavirus strain, is characterised by a short electropherotype and G2P[4] serotype specificity. Following sequence-independent genome amplification and 454[®] pyrosequencing of genomic cDNA, differences between the newly determined consensus sequence and GenBank sequences were observed in 10 of the 11 genome segments. Only the consensus sequence of genome segment 1 was identical to sequences deposited in GenBank. A novel isoleucine at position 397 in a hydrophobic region of VP4 is described. An additional 7 N-terminal amino acids was found in NSP1. For genome segment 10 the first 34 and last 30 nucleotides of the 5' and 3'-terminal ends, respectively, were identified. Genome segment 11 was found to be 821 bp long, which is 148 bp longer than the full length genome segment 11 sequence reported previously. This paper reports the first complete consensus genome sequence for the tissue culture adapted DS-1 strain free from cloning bias and the limitations of Sanger sequencing. Sequence differences in previous publications reporting on DS-1 rotavirus genome segment sequencing, were identified and discussed.

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Rotaviruses belong to the *Reoviridae* family and are the most common cause of severe childhood diarrhoea worldwide (Parashar et al., 2006). The triple layered virus particle contains a genome of 11 dsRNA segments. The genome encodes six structural proteins (VP1–VP4, VP6 and VP7) and six non-structural proteins (NSP1–NSP6) (Estes and Kapikian, 2007). Rotavirus genetic diversity is generated through the accumulation of point mutations, genome segment reassortment and intragenic recombination during mixed infections (Parra et al., 2004; Ramig, 1997; Taniguchi and Urasawa, 1995). Rearrangement downstream of the open reading frame (ORF) can occur *in vitro* in tissue culture when rotavirus is propagated at a high multiplicity of infection and also *in vivo* in immune deficient children (Hundley et al., 1987; Pedley et al., 1984). Matthijnsens et al. (2006) proposed that the rearrangement mechanism involves re-entry of the 3'-end of the negative strand into the catalytic core (forming a loop) and the RNA-dependent RNA polymerase making a mistake by switching template.

The DS-1 strain (G2P[4] serotype) is the prototype of DS-1-like strains (subgroup I, short electropherotype). The strain was

isolated in 1976 in Washington D.C. (USA) from a gastroenteritis patient (Kalica et al., 1981). Primary adaptation from stool, of the slow-growing strain was performed in MA104 cells without infection of primary cells (Wyatt et al., 1983). Sequence differences exist between DS-1 rotavirus genome segments determined previously. A full genome sequence for the DS-1 strain was reported by Heiman et al. (2008). However, the extreme 5' and 3' termini could not be determined directly as genome segment-specific terminal primers were used for RT-PCR amplification. To date, no consensus sequence, which represents the most viable and predominant population of the viral quasispecies (Domingo et al., 2006) has been determined for the DS-1 genome. In this study the complete consensus sequence for the DS-1 genome was determined using sequence-independent amplification and 454[®] pyrosequencing (Margulies et al., 2005).

The culture-adapted DS-1 virus was provided at 5×10^5 ffu/ml by Dr. Carl Kirkwood (Murdoch Children's Research Institute, Melbourne, Australia) after it had been propagated 10 times in MA104 cells (each propagation at an m.o.i. of <0.5) following initial adaptation. In this study the virus stock was activated with 10 µg/ml porcine trypsin IX (Sigma) prior to infecting confluent MA104 cells. For dsRNA extraction, confluent MA104 cells in a 75 cm² flask were infected at a low m.o.i. of <1 and propagated in Dulbecco's modified essential medium (Hyclone) containing 1 µg/ml porcine trypsin IX, 1% non essential amino acids (Gibco) and

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Table 1Summary of the DS-1 consensus sequence data determined with 454[®] pyrosequencing in comparison to DS-1 GenBank sequences.

| Genome segment | Size (bp) | Amino acids in coding region | Nucleotide differences | | | Amino acid differences | |
|----------------|-----------|------------------------------|------------------------|----------------|-----------------|------------------------|---|
| | | | Insertions | Deletions | Point mutations | Insertions | Point mutations |
| 1 (VP1) | 3302 | 1088 | None | None | None | None | None |
| 2 (VP2) | 2684 | 879 | None | None | 3 | None | None |
| 3 (VP3) | 2591 | 835 | None | None | 11 | None | None |
| 4 (VP4) | 2359 | 775 | None | None | 28 | None | ^a 52S→H, 53W→G, 106S→I, 107S→A, 142T→M, 144T→K, 150N→D, 230R→S, 245K→R, 280I→V, 380T→I, and 397N/D→I |
| 5 (NSP1) | 1563 | 493 | 1 | ^b 2 | 5 | 1–7 MKSLVEA | ^c 137 S→L, 338 I→T, 381 E→D, 488R→G |
| 6 (VP6) | 1356 | 397 | None | None | 1 | None | None |
| 7 (NSP3) | 1064 | 313 | None | None | 1 | 1–3 MLK | ^d 51G→D |
| 8 (NSP2) | 1059 | 317 | None | None | 1 | None | 194G→D |
| 9 (VP7) | 1062 | 326 | None | None | 5 | None | None |
| 10 (NSP4) | 751 | 175 | None | None | None | None | None |
| 11 (NSP5) | 821 | 200 | 6 | None | 6 | 139–140QE | ^e 9G→S and 49N→R |

^a Differences in the translated VP4 amino acid sequence: residues 52–150 occur in VP8* region; residue 150 is a glycosylation site; residue 230 is a trypsin cleavage site; residue 380 occurs in an antigenic region and residue 397 in a hydrophobic region.

^b Deletion of TA at nucleotides 1553–1554 in the 3'-UTR.

^c Differences in translated NSP1 amino acid sequence: residue 137 occurs in a region that is important for cytoskeletal localisation while the other changes occur in a region that interacts with interferon regulatory factor 3.

^d Amino acid difference in translated NSP3 occurs in an RNA-binding region.

^e The 9G→S difference in the translated NSP5 amino acid sequence occurs in a region that interacts with VP1.

1% antibiotics (penicillin/streptomycin/amphotericin) (Lonza) for 5 days. Viral dsRNA was extracted using Trizol reagent (Invitrogen) and single-stranded RNA precipitated with 2 M LiCl (Sigma) at 4 °C for 16 h followed by centrifugation at 16 000 × g for 30 min. The dsRNA in the supernatant was purified using a MinElute kit (Qiagen) following the manufacturer's instructions. To obtain a dataset free from cloning bias, sequence-independent genome amplification was performed as described by Potgieter et al. (2009) with the following modifications: genomic cDNA was synthesised with Transcriptor High Fidelity reverse transcriptase (Roche) and amplified using Phusion High Fidelity DNA polymerase (Finnzymes). Amplified cDNA was purified with a QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions. The 454[®] pyrosequencing was performed at Inqaba Biotec[™], South Africa, using GS FLX Titanium (Roche).

Pyrosequence data comprised of 10 180 sequence reads of approximately 400 bp each. The Lasergene[™] 8.1.2 SeqMan Pro (DNASTAR[®]) was used for sequence assembly. A total of 36 contigs were obtained and used to determine the complete consensus sequence (Supplementary data S1) for each genome segment. The total size of the consensus genome obtained was 18 612 bp. The average depth of coverage was 204-fold, with genome segment 1 displaying the lowest (22-fold) and genome segment 8 the highest (458-fold) coverage. The identified consensus sequence for each genome segment (GenBank IDs in Supplementary data S2) was compared to all DS-1 rotavirus genome sequences in GenBank (Supplementary data S2).

The termini of the 5' untranslated regions (UTRs) for all genome segments were the same as described previously for rotaviruses, i.e., 5'-GGC(U/A)₇ (Tortorici et al., 2006). The 3' tetranucleotide terminal sequence GACC-3' was also obtained as expected (Wentz et al., 1996). The 3'-terminal heptanucleotide sequence, UGUGACC-3', was present in 9 genome segments, but in genome segments 2 and 10 it was UAUGACC-3'. The UAUGACC-3' sequence was reported before for the DS-1 strain in genome segment 2 (Matthijnssens et al., 2008). Using the RotaC classification tool

(Maes et al., 2009), the full genotype of the DS-1 rotavirus strain was confirmed as G2-P[4]-I2-R2-C2-M2-A2-N2-T2-E2-H2.

Differences were observed between the consensus DS-1 sequences and DS-1 sequences in GenBank in 10 of the 11 genome segments. Only the consensus sequence of genome segment 1 (VP1) was identical to the GenBank genome segment 1 sequences. While nucleotide differences were observed in genome segments 2 (VP2), 3 (VP3), 6 (VP6), and 9 (VP7), there were no associated amino acid changes (Table 1). Amino acid differences were observed in deduced amino acid sequences of VP4, NSP1, NSP2, NSP3 and NSP5 (Table 1). In the deduced NSP2 amino acid sequence, a 194G→D change was observed while a 51D→G change was observed in an RNA-binding region of the NSP3 amino acid sequence. A GenBank sequence, EF672579, encoding NSP3 lacks MLK, the first three residues.

The consensus sequence for genome segment 4 (VP4) was compared to four GenBank sequences AB118025, AJ540227, EF672577 and DQ141310. The consensus sequence was identical to DQ141310 (nucleotides 186–678 in the consensus sequence). A 2% variation resulted from a total of 28 nucleotide differences (Supplementary data S3) between all the genome segment 4 sequences. Twelve of these nucleotide differences resulted in amino acid changes. Changes observed in the VP8* region of VP4 were 52S→H, 53W→G, 106S→I, 107S→A, 142T→M, 144T→K, 150N→D and 230R→S. Close scrutiny of the VP8* structure (PDB ID: 2AEN; Monnier et al., 2006) indicated that I106 and A107 are inside the VP8* structure while M142, K144 and D150 are located on the surface of the VP8* structure. A 245K→R change was observed in the region between VP8* and VP5*. Amino acid differences 280I→V and 380T→I were observed in the antigenic domain of VP5*. Furthermore, a novel isoleucine at position 397 (in a hydrophobic region) was observed. Residues V280, I380 and I397 were located on the surface of the predicted VP5* structure (based on PDB ID: 1SLQ; Dormitzer et al., 2004). The novel I397 increases hydrophobicity in the loop region and may increase infectivity since hydrophobicity of the VP5* apex is required for membrane disruption.

| | | | | | | | | | | | | | | | | | |
|-----------------|-----------------|----------------|---------------|---------------|--------------|--------------|--------------|--------------|--------------|----------------|---------------|--------------|---------------|--------------|-------------|-------------|---------------|
| | | | | | 20 | | | | | 40 | | | | | 60 | | |
| DS-1 NSP1 CS | MKSLVEA | MAT | FKDAC | YQYK | LNKL | NAV | LK | LGAND | VWR | PS | TLTKR | KGW | CL | DCCQ | HTD | LT | TY |
| EF672578_DS-1 | | | | | | | | | | | | | | | | | |
| L18945_DS-1 | | | | | | | | | | | | | | | | | |
| GER1H-09 strain | | | | | | | | | | | | | | | | | |
| N26-02 strain | | | | | | | | | | | | | | | | | |
| B1711 strain | | | | | | | | | | | | | | | | | |
| | | | | | 80 | | | | | 100 | | | | | 120 | | |
| DS-1 NSP1 CS | CQGCL | IYHVC | EWCSQ | YNRCF | LDDDP | HLLRM | RTFRN | EITKS | DLENL | INMYN | TLFP | INKK | IV | | | | |
| EF672578_DS-1 | | | | | | | | | | | | | | | | | |
| L18945_DS-1 | | | | | | | | | | | | | | | | | |
| GER1H-09 strain | | | | | | | | | | | | | | | | | |
| N26-02 strain | | | | | | | | | | | | | | | | | |
| B1711 strain | | | | | | | | | | | | | | | | | |
| | | | | | 140 | | | | | 160 | | | | | 180 | | |
| DS-1 NSP1 CS | HKFANT | IKQH | KCRNEY | TQW | YNHFL | MPI | ITL | QSLSI | ELDGD | IYYIF | GYDD | MHKIN | QTPFS | | | | |
| EF672578_DS-1 | | | | | | | | | | | | | | | | | |
| L18945_DS-1 | | | | | | | | | | | | | | | | | |
| GER1H-09 strain | N | | | | | | | | | | | | | | | | |
| N26-02 strain | N | | | | | | | | | | | | | | | | |
| B1711 strain | N | A | | | | | | | | | | | | | | | |
| | | | | | 200 | | | | | 220 | | | | | 240 | | |
| DS-1 NSP1 CS | FTNL | ISKYDM | LLD | SINFDR | MAFLP | LT | LQ | EYALR | YFSKS | RFITERR | KCI | EILH | FS | DNIL | | | |
| EF672578_DS-1 | | | | | | | | | | | | | | | | | |
| L18945_DS-1 | | | | | | | | | | | | | | | | | |
| GER1H-09 strain | | | | | | | | | | | | | | | | | |
| N26-02 strain | | | | | | | | | | | | | | | | | |
| B1711 strain | | | | | | | | | | | | | | | | | |
| | | | | | 280 | | | | | 300 | | | | | 320 | | |
| DS-1 NSP1 CS | DNLHNP | NFTL | QVIRN | CSNMS | VEWNK | ACN | I | RN | ISDY | FDIL | KSSHTE | FYNI | SPRCRM | F | FTQY | | |
| EF672578_DS-1 | | | | | | | | | | | | | | | | | |
| L18945_DS-1 | | | | | | | | | | | | | | | | | |
| GER1H-09 strain | ND | | | | | | | | | | | | | | | | |
| N26-02 strain | ND | | | | | | | | | | | | | | | | |
| B1711 strain | ND | | | | | | | | | | | | | | | | |
| | | | | | 320 | | | | | 340 | | | | | 360 | | |
| DS-1 NSP1 CS | KLKI | ASKLIK | P | NYVAS | NHNS | LATE | VH | NCKW | CS | INNS | T | VW | NDFRI | K | NVYN | DIFN | FIRALV |
| EF672578_DS-1 | | | | | | | | | | | | | | | | | |
| L18945_DS-1 | | | | | | | | | | | | | | | | | |
| GER1H-09 strain | | | | | | | | | | | | | | | | | |
| N26-02 strain | | | | | | | | | | | | | | | | | |
| B1711 strain | | | | | | | | | | | | | | | | | |
| | | | | | 380 | | | | | 400 | | | | | 420 | | |
| DS-1 NSP1 CS | KSNLY | VGHCS | SEEKI | YESIK | DVLNV | CKENE | WNMLV | TE | MFN | QLEPI | KL | NEN | NYILL | NYEIN | | | |
| EF672578_DS-1 | | | | | | | | | | | | | | | | | |
| L18945_DS-1 | | | | | | | | | | | | | | | | | |
| GER1H-09 strain | | | | | | | | | | | | | | | | | |
| N26-02 strain | | | | | | | | | | | | | | | | | |
| B1711 strain | | | | | | | | | | | | | | | | | |
| | | | | | 440 | | | | | 460 | | | | | 480 | | |
| DS-1 NSP1 CS | WV | MNV | LINS | IGKIP | KILT | LDVIL | LIRII | IYD | W | FDIR | FM | RNT | PMTT | FTV | NK | LQ | LYEKD |
| EF672578_DS-1 | | | | | | | | | | | | | | | | | |
| L18945_DS-1 | | | | | | | | | | | | | | | | | |
| GER1H-09 strain | | | | | | | | | | | | | | | | | |
| N26-02 strain | | | | | | | | | | | | | | | | | |
| B1711 strain | | | | | | | | | | | | | | | | | |
| | | | | | 480 | | | | | 493 | | | | | 493 | | |
| DS-1 NSP1 CS | RTAEHDS | GIS | DIE | | | | | | | | | | | | | | |
| EF672578_DS-1 | | | | | | | | | | | | | | | | | |
| L18945_DS-1 | | | | | | | | | | | | | | | | | |
| GER1H-09 strain | | | | | | | | | | | | | | | | | |
| N26-02 strain | | | | | | | | | | | | | | | | | |
| B1711 strain | | | | | | | | | | | | | | | | | |

Fig. 1. Alignment of the deduced NSP1 amino acid consensus sequence (DS-1 NSP1 CS) with DS-1 sequences (EF672578 and L18945) and selected DS-1-like sequences (GER1H-09, N26-02 and B1711) in GenBank. The first 7 residues, MKSLVEA, observed at the N-terminal end of the translated consensus sequence and not in GenBank DS-1 sequences but present in the DS-1-like strains B1711, G9P[G]; GER1H-09, G8P[G]; and N26-02, G12P[G] are boxed and differences between the deduced consensus sequence and the DS-1 sequences are shaded. The conserved DS-1 cysteine-rich zinc finger motif is underlined.

tion during rotavirus cell entry (Kim et al., 2010). Pyrosequence data showed that at the corresponding codon position encoding amino acid residue 397, 30% (approximately 150 reads) of sequence reads indicated an AAT codon (Supplementary data S4) encoding an asparagine residue. The 30% translated sequence reads containing N397 suggests that a quasispecies population exists within the analysed DS-1 strain.

VP8* is an important target for neutralising antibodies and P serotype specificity (Dormitzer et al., 2002). The region from amino acids 106–159 is generally a variable region (Gorziglia et al., 1986). The probable biological effect of residues 142 and 144 may be related to antigenicity since these residues are located on the surface of the protein structure in a hypervariable region. According to published sequencing reports, residue 150 is an N-linked glycosy-

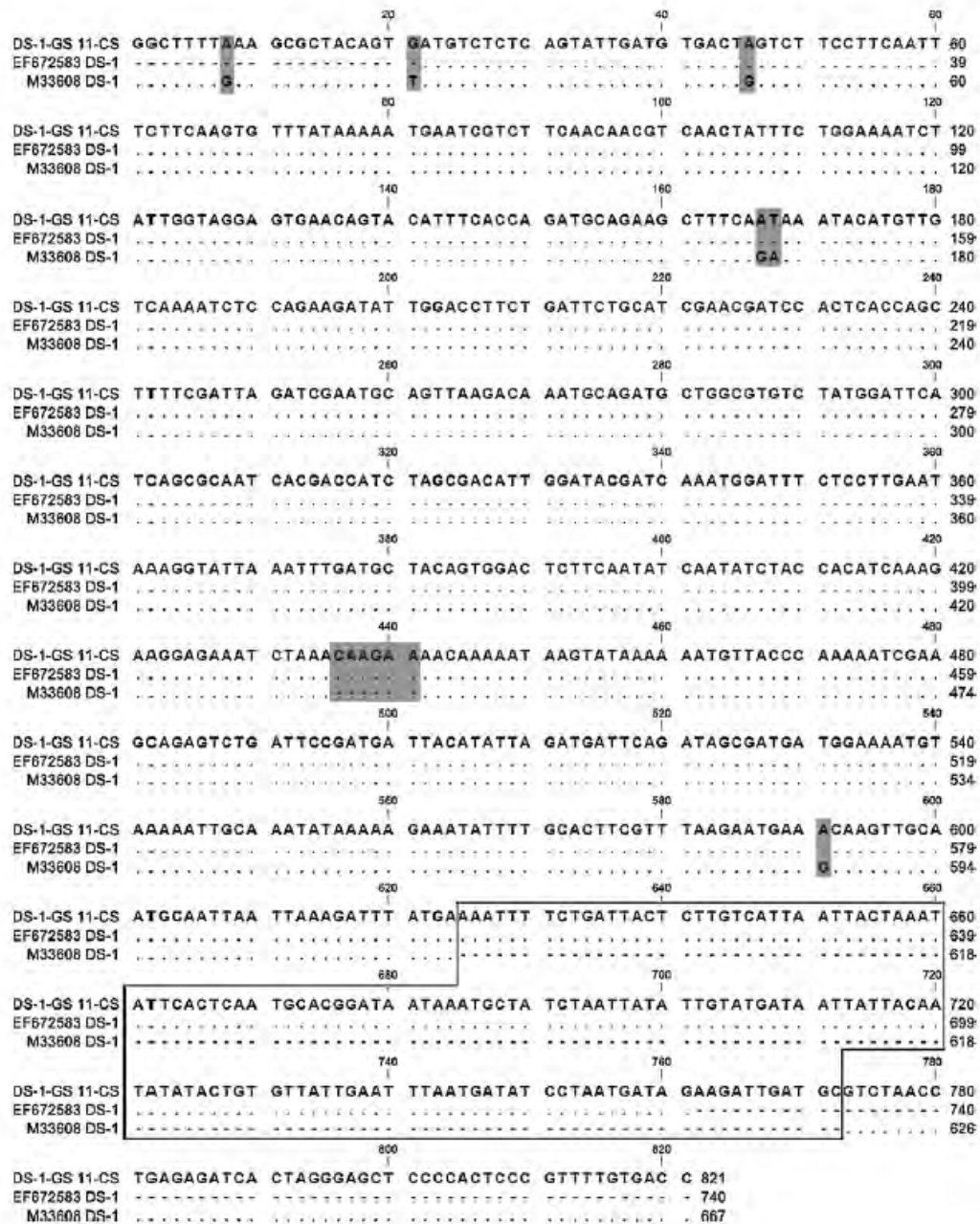


Fig. 2. Alignment of genome segment 11 consensus nucleotide sequence (DS-1-GS 11-CS) with DS-1 genome segment 11 sequences from GenBank (M33608 and EF672583). Boxed sequences (nucleotides 625–772) indicate the 148-nucleotide deletion in M33608. Nucleotide changes and a 6-base deletion in M33608 at positions 436–441 are shaded.

lation site in the DS-1 strain. However, a 150N→D change observed in this study indicates that this site is not glycosylated in the DS-1 strain analysed. This change may affect efficiency of cell entry, antigenicity and virulence (Chattopadhyay et al., 2010) and should be investigated *in vivo*.

Trypsin cleavage of VP4 occurs between R230 and N231 (Arias et al., 1996). However, in this study a 230R→S change was observed. A second trypsin cleavage site present between R246 and A247 was unchanged in the consensus sequence. Cleavage at either or both cleavage sites results in the conformational changes required for entry into cells (Yoder et al., 2009). The cleavage site between R246 and A247 may be preferred for cleavage in the DS-1 strain.

Cleavage site preference was observed in the SA11 rotavirus strain where R247 is preferred to R241 (R230 equivalent in DS-1 strain) (Gorziglia et al., 1986). While the biological effect of differences at trypsin cleavage sites is not clear, a second trypsin cleavage site in VP4 has been correlated with virulence (Estes and Cohen, 1989).

For genome segment 5 (NSP1), the ORF of the consensus sequence was 21 nucleotides longer at the 5'-end, than the ORFs of GenBank sequences L18945 and EF672578. The consensus DS-1 NSP1 amino acid sequence therefore contained 7 additional amino acids (MKSLVEA) at the N-terminal end when compared to L18945 and EF672578 (Fig. 1). This suggests that genome segment 5 might have an alternative start codon. NSP1 is known to vary in length

among different rotavirus strains. For instance, NSP1 of the Wa strain is 486 amino acids long while that of SA11 is 495 amino acids (Nakagomi and Kaga, 1995). The DS-1 NSP1 deduced from the consensus nucleotide sequence was 493 amino acids long. Alignment of the DS-1 consensus sequence to DS-1-like strains such as GER1H-09, N26-02 and B1711 showed that their first 7 N-terminal residues were identical. The full genotype assignment showed that these DS-1-like strains differ only at the P and G genotypes from the DS-1 full genotype. However, published sequences for other DS-1-like strains such as the Tb-Chen strain (G2P[4]) do not have the MKSLVEA sequence at the N-terminal end of NSP1. The most important functional feature of NSP1 is the highly conserved cysteine-rich zinc finger motif (Graff et al., 2007) which was conserved as expected (Fig. 1).

The 34 5'-terminal and 30 3'-terminal nucleotide sequences of genome segment 10 (NSP4) have not been reported previously. These nucleotide sequences were determined in this study as 5'-GGCTTTTAAAGATTCTGTTCCGAGAGAGCGGTG-3' at the 5' terminus, and 5'-GTTAATGGAAGGACGGTCTTAATATGACC-3' at the 3' terminus.

The consensus sequence for genome segment 11 (NSP5) obtained in this study was 821 bp (Fig. 2) and was compared to a full length DS-1 genome segment 11 sequence (GenBank ID: M33608) of 667 bp. A 148 bp nucleotide sequence (nucleotides 625–772) was not present in the 3'-UTR of M33608, while a GenBank sequence EF672583, a partial DS-1 genome segment 11 sequence, lacks 21 nucleotides at the 5' terminus and 60 nucleotides at the 3' terminus (Fig. 2). No internal duplications were observed in the consensus genome segment 11 sequence, indicating the absence of genome segment rearrangement. The 148 bp region may modify the formation of stem loops or cruciform structures (Supplementary data S5) in the dsRNA that have structural or functional roles. Six potentially *cis*-acting 5'-CUUC motifs (Li et al., 2010) were present in M33608 and the consensus sequence at nucleotides 49–52, 53–56, 62–65, 89–92, 206–209, 392–395. A seventh 5'-CUUC motif is present at nucleotides 568–571 in M33608 and at nucleotides 574–577 in the consensus sequence. A potentially *cis*-acting 5'-GGGAGCUCC palindrome (Li et al., 2010) spanned nucleotides 794–803 in the consensus genome segment 11 sequence and is present at nucleotides 640–649 in M33608 (Supplementary data S5).

In conclusion, the application of the sequence-independent genome amplification technology in combination with 454[®] pyrosequencing of the DS-1 genome allowed the presentation of a consensus sequence for this strain free from cloning-bias and the limitations of Sanger sequencing. In the process, sequence differences in genome segments 2–11 that were observed during previous sequencing efforts, were addressed. The results suggest the occurrence of quasispecies in some genome segments of the tissue culture-adapted DS-1 strain such as genome segment 4. It is possible that some of the differences reported here resulted from cell culture propagation of the DS-1 strain in different laboratories, introducing point mutations into the genome.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jviromet.2011.05.004.

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