**Figure S1.** Example of an RT-qPCR analysis of an SA11 NSP3-encoding plasmid standard for the determination of genome copy equivalents (GCEs)/ml culture supernatant. A plasmid preparation of pT7-NSP3SA11 with a known number of GCEs ($2.34 \times 10^{11}$ copies/µg) was serially diluted and 5 µl were used in RT-qPCR analyses in parallel with 5 µl RNA isolated from culture supernatants of infected MA-104 cells. Linear regression analysis of the RT-qPCR results of the plasmid standard resulted in a formula enabling the calculation of GCEs contained in 5 µl RNA on the basis of the Ct values. The GCEs/ml culture supernatant were calculated by multiplying the number of GCEs contained in 5 µl RNA with factor A (to account for the volume of culture supernatant that RNA was prepped from), factor B (to account for the RNA elution buffer volume), and factor C (to account for the dilution due to DNase digest).
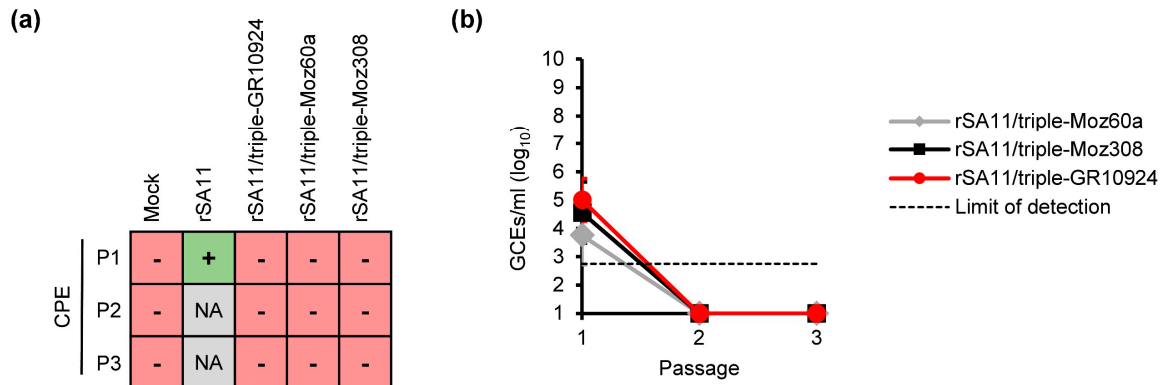
**Figure S2.** Repetition experiments for the generation of SA11 triple-reassortants containing VP4, VP7 and VP6 from three African human RVA strains. **(a)** Overview of the developed cytopathic effect (CPE) upon passaging in MA-104 cells. Data are representative of two independent experiments. **(b)** Analyses of the freeze-thaw supernatants by qRT-PCR after each passage in MA-104 cells. Data are means ± standard deviation from two independent experiments. Mock = Mock-infected cells; rSA11 = Recombinant SA11; rSA11/triple-GR10924, rSA11/triple-Moz60a and rSA11/triple-Moz308 = Recombinant rotaviruses carrying segment 4 (VP4), segment 9 (VP7) and segment 6 (VP6) from the indicated human RVA strain in the backbone of SA11; P1-3 = Passages 1-3; red minus = No CPE; green plus = Strong CPE; NA = Not analyzed; GCEs = Genome copy equivalents.
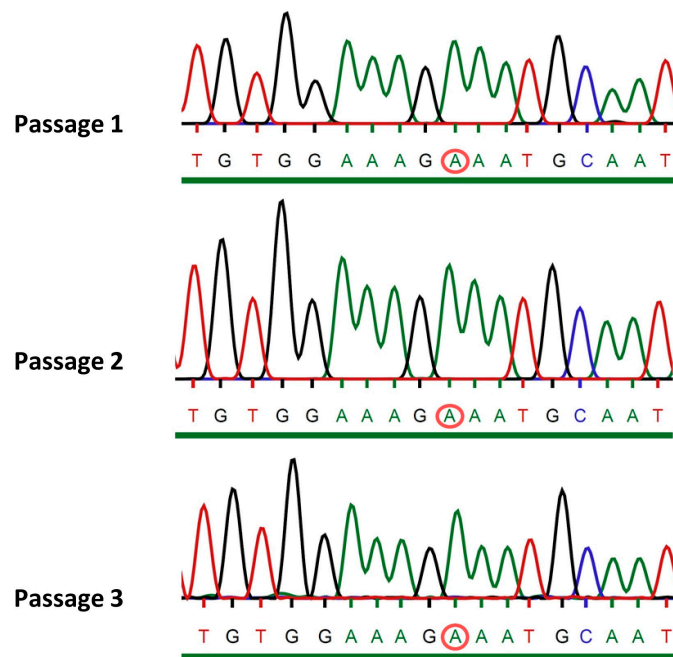
Passage 1

Passage 2

Passage 3

TGTGGAAAGAAATGCAAT
TGTGGAAAGAAATGCAAT
TGTGGAAAGAAATGCAAT

**Figure S3.** Sanger sequencing analyses of the VP4-encoding genome segment from the rSA11/triple-GR10924 reassortant that replicated to a higher titer in early passages. Sequencing chromatograms of passages 1-3 are shown. The read circle marks nucleotide position 797 in the VP4-encoding genome segment from human RVA strain GR10924. The green line below the chromatograms indicates that the probability for a wrong base call was equal to or less than 1 in 1000.
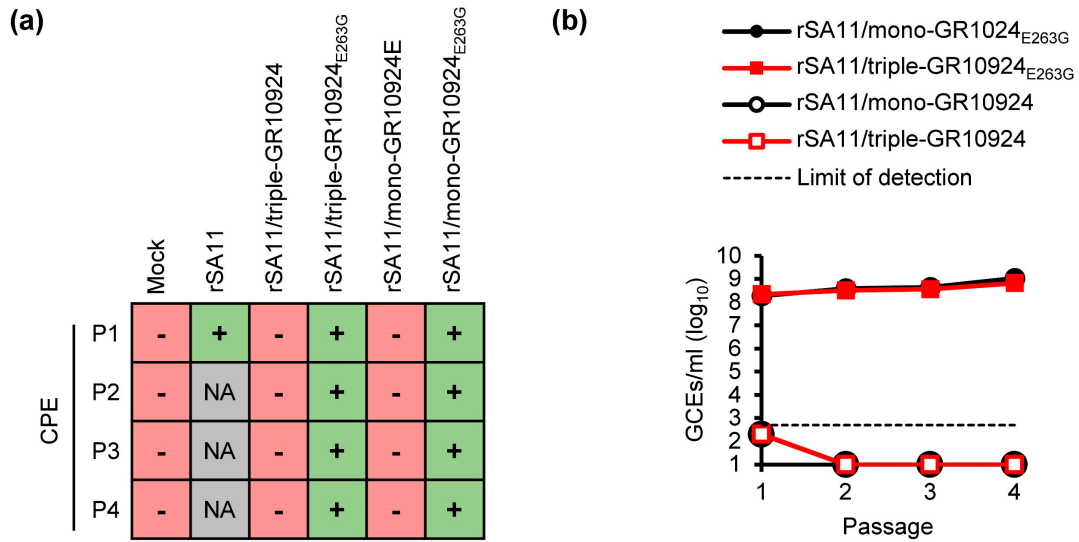
**(a)**

| CPE | Mock | rSA11 | rSA11/triple-GR10924 | rSA11/triple-GR10924$_{E263G}$ | rSA11/mono-GR10924E | rSA11/mono-GR10924$_{E263G}$ |
|---|---|---|---|---|---|---|
| P1 | - | + | - | + | - | + |
| P2 | - | NA | - | + | - | + |
| P3 | - | NA | - | + | - | + |
| P4 | - | NA | - | + | - | + |

**(b)**

- ●— rSA11/mono-GR1024$_{E263G}$
- ■— rSA11/triple-GR10924$_{E263G}$
- ○— rSA11/mono-GR10924
- □— rSA11/triple-GR10924
- ------- Limit of detection

**Figure S4.** Repetition experiment for the rescue of SA11 mono- and triple-reassortants containing VP4 from human RVA strain GR10924 with and without the mutation A797G in VP4. **(a)** Overview of the developed cytopathic effect (CPE) upon passaging in MA-104 cells. **(b)** Analyses of the freeze-thaw supernatants by qRT-PCR after each passage in MA-104 cells. Mock = Mock-infected cells; rSA11 = Recombinant SA11; rSA11/triple-GR10924 = Recombinant rotavirus carrying segment 4 (VP4), segment 9 (VP7) and segment 6 (VP6) from human RVA strain GR10924 in the backbone of SA11; rSA11/triple-GR10924$_{E263G}$ = rSA11/triple-GR10924 with VP4-E263G; rSA11/mono-GR10924 = Recombinant rotavirus carrying segment 4 (VP4) from human RVA strain GR10924 in the backbone of SA11; rSA11/mono-GR10924$_{E263G}$ = rSA11/mono-GR10924 with VP4-E263G; P1-4 = Passages 1-4; red minus = No CPE; green plus = Strong CPE; NA = Not analyzed; GCE = Genome copy equivalents.
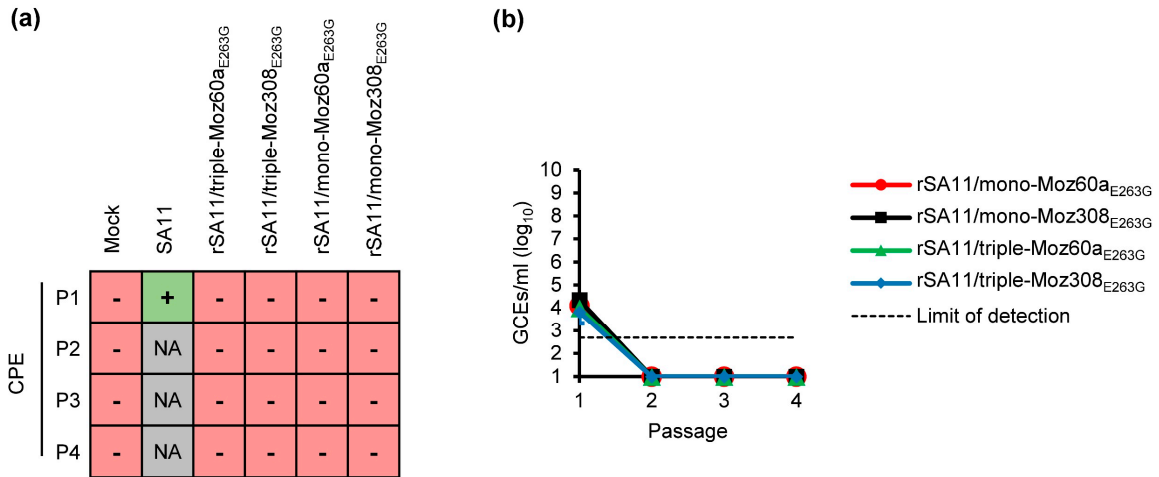
**Figure S5.** Attempts to rescue of SA11 mono- and triple-reassortants containing VP4 from human RVA strains Moz60a or Moz308 with the mutation A797G in VP4. **(a)** Cytopathic effect (CPE) upon passaging in MA-104 cells. The result is representative of one experiment performed in duplicates. **(b)** Determined number of GCEs/ml in freeze-thaw supernatants after each passage. Data are means ± standard deviation from one experiment performed in duplicates. rSA11/triple-Moz308$_{E263G}$, rSA11/triple-Moz60a$_{E263G}$ = Recombinant rotaviruses carrying segment 4 (VP4) with VP4-E263G, segment 9 (VP7) and segment 6 (VP6) from the indicated human RVA strain in the backbone of SA11; rSA11/mono-Moz308$_{E263G}$, rSA11/mono-Moz60a$_{E263G}$ = Recombinant rotaviruses carrying segment 4 (VP4) with VP4-E263G from the indicated human RVA strain in the backbone of SA11; P1-4 = Passages 1-4; red minus = No CPE; green plus = Strong CPE; NA = Not analyzed.
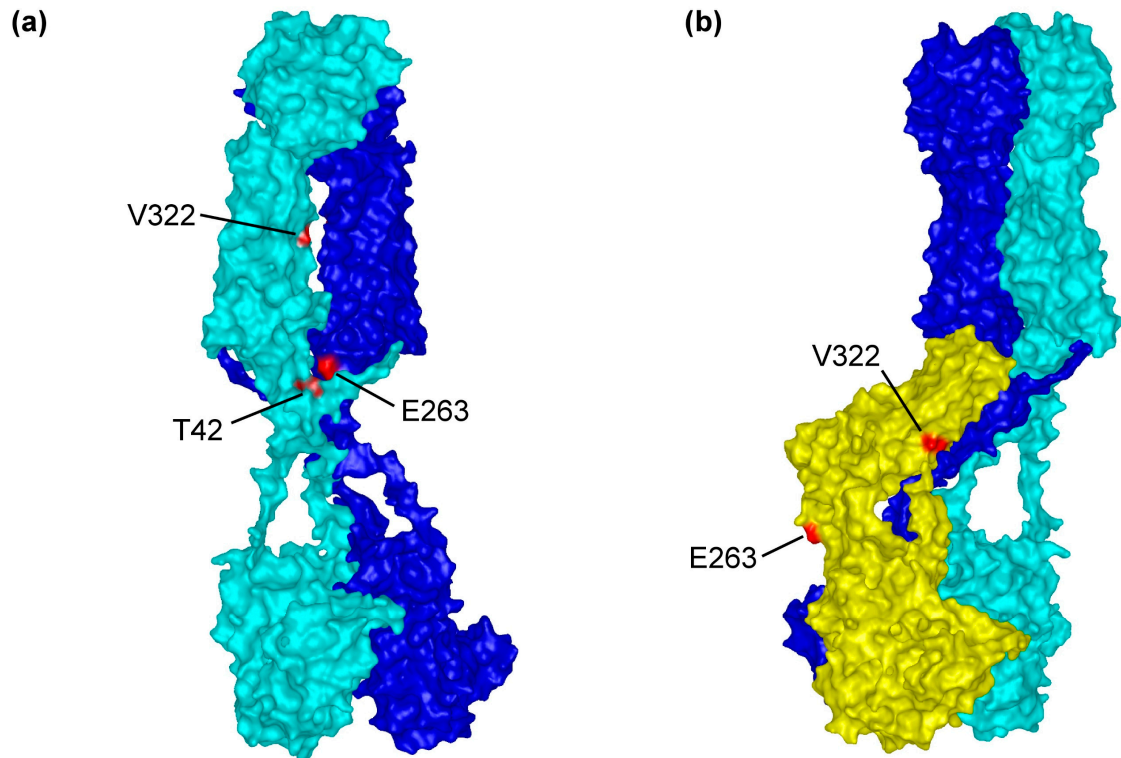
**Figure S6.** Mapping of E263, T42 and V322 to the VP4 trimeric spike. The three-dimensional structure of the VP4 spike on the basis of the atomic model of an infectious rhesus rotavirus particle (PDB 4v7q, chains BX, BY and BZ) is shown. VP4A, VP4B and VP4C are colored in blue, cyan and yellow, respectively. **(a)** The position of the amino acid residue corresponding to E263, T42 and V322 in VP4A and VP4B from GR10924 are highlighted in red. **(b)** The position of the amino acid residue corresponding to E263 and V322 in VP4C from GR10924 are highlighted in red.
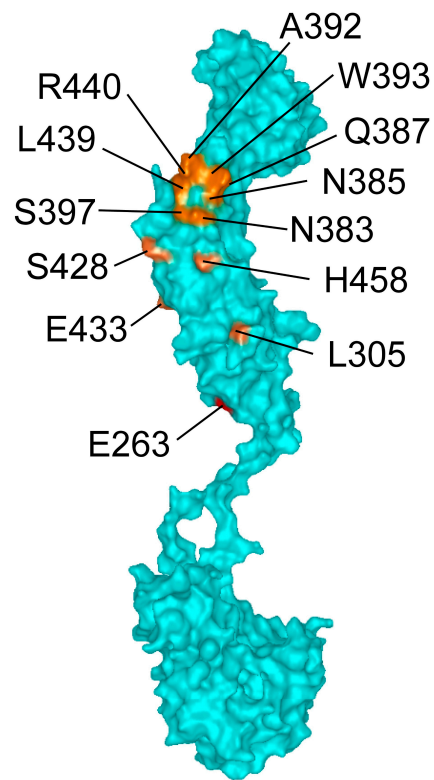
**Figure S7.** Mapping of known VP5* antigenic epitopes to the three-dimensional structure of VP4 from rhesus rotavirus (PDB 4v7q, chain BX). Antigenic epitopes are highlighted in orange and the amino acid residue as well as the position corresponding to VP4 from GR10924 are shown. The amino acid residue corresponding to E263 in VP4 from GR10924 is highlighted in red.

**Table S1.** Primer pairs used for RT-PCR analyses, Sanger sequencing and mutagenesis.

| Primer | Sequence | Figure |
|---|---|---|
| VP4 GR10924-seq-f | 5'-TGGGTTACCACCAATGCAGAA-3' | 2b |
| VP4 GR10924-seq-r | 5'-CACACCATTGACTGAGCAAGT-3' | 2b |
| VP2 SA11-f | 5'-CGTAATAGATGGTATTGGAAATT-3' | 3c |
| VP2 SA11-r | 5'-CTGGTATGTAATTGAAAATGATA-3' | 3c |
| VP4 SA11-f | 5'-ACGACAGTTGGACCATTGCT-3' | 3c |
| VP4 SA11-r | 5'-AGCGTTAGGTGTCTGTCCTTC-3' | 3c |
| VP7 SA11-f | 5'-TATTATCCGACTGAGGCTGCG-3' | 3c |
| VP7 SA11-r | 5'-GCAACGTCGCGTCATATTTCA-3' | 3c |
| VP4 GR10924-f | 5'-GATGAGTGGAGGTGCAGTGT-3' | 3c |
| VP4 GR10924-r | 5'-GCTGGTAGCCCGTACAATCC-3' | 3c |
| VP7 GR10924-f | 5'-ACCGATGTTGTTGATGGTGTG-3' | 3c |
| VP7 GR10924-r | 5'-ACATCTGAGCCACCGACTTG-3' | 3c |
| VP6-seq-f | 5'-CAAATGATAATTACTATGAATGGAAATGA-3' | 3d |
| VP6-seq-r | 5'-TAGAAACTCCACTTCAACGTTATT-3' | 3d |
| VP4mut GR10924-f | 5'-TGTGGAAAGGAATGCAATATAACAGAG-3' | n.s. |
| VP4mut GR10924-r | 5'-ACGATGTCTTTGATATAATGATATCCTCAT-3' | n.s. |
| VP4mut Moz60a-f | 5'-TATGGAAAGGAATGCAATATAACAGAGAT-3' | n.s. |
| VP4mut Moz60a-r | 5'-ATGAAGTCTTTGAAATTGTAATGTCTTCATT-3' | n.s. |
| VP4mut Moz308-f | 5'-TATGGAAGGGAATGCAATATAATAGAGATATT-3' | n.s. |
| VP4mut Moz308-r | 5'-ATGAGGTTTTTGAAATTGTAATATCTTCATTAACT-3' | n.s. |
| VP4 Moz60a-seq-f | 5'-TCCAAGGTCCCAAGAGTCTAA-3' | n.s. |
| VP4 Moz60a-seq-r | 5'-GGTGGGTAAAGATCCTCCGT-3' | n.s. |
| VP4 Moz308-seq-f | 5'-ACCACTGATAGATCAGATACTGC-3' | n.s. |
| VP4 Moz308-seq-r | 5'-GTAATTTGTTCACCATCACGCA-3' | n.s. |

n.s. – not shown

**Table S2**. Average coverage, coverage range, and % ORF sequenced of rSA11/triple-GR10924 and rSA11/triple-GR10924 duplicate after next generation sequencing of virus present in the supernatant of the 10<sup>th</sup> passage.

| Segment | rSA11/triple-GR10924 | | | rSA11/triple-GR10924 duplicate | | |
|---|---|---|---|---|---|---|
| | Average coverage | Coverage range | % ORF sequenced | Average coverage | Coverage range | % ORF sequenced |
| NSP1 | 349 | 110-495 | 100.0 | 807 | 192-1169 | 100.0 |
| NSP2 | 281 | 161-390 | 100.0 | 603 | 242-865 | 100.0 |
| NSP3 | 299 | 97-397 | 100.0 | 537 | 154-713 | 100.0 |
| NSP4 | 177 | 63-250 | 100.0 | 247 | 85-380 | 100.0 |
| NSP5 | 122 | 46-157 | 100.0 | 222 | 54-278 | 100.0 |
| VP1 | 233 | 20-325 | 100.0 | 528 | 109-723 | 100.0 |
| VP2 | 224 | 34-361 | 100.0 | 508 | 87-727 | 100.0 |
| VP3 | 234 | 69-363 | 100.0 | 528 | 201-763 | 100.0 |
| VP4 | 205 | 4-345 | 100.0 | 499 | 2-664 | 99.9[A] |
| VP6 | 292 | 68-464 | 100.0 | 573 | 83-882 | 100.0 |
| VP7 | 318 | 135-468 | 100.0 | 555 | 165-758 | 100.0 |

[A]First two nucleotides of the VP4 ORF missing

**Table S3.** UTRs of the genome segments encoding structural viral proteins from rSA11/triple-GR10924 and rSA11/triple-GR10924 duplicate. Accession numbers of reference sequences are shown in brackets. % UTR = percentage of the UTR identified by sequencing in relation to the reference sequence; NA = Not applicable.

| Segment | Sequence 5' -> 3' | % UTR |
|---|---|---|
| VP1_5'UTR (LC178564) | GGCTATTAAAGCTGTACA | NA |
| rSA11/triple-GR10924 | AAGCTGTACA | 55.6 |
| rSA11/triple-GR10924 duplicate | AAGCTGTACA | 55.6 |
| VP1_3'UTR (LC178564) | AACGCTTAGATGTGACC | NA |
| rSA11/triple-GR10924 | AACGCTTAGATGTGA | 88.2 |
| rSA11/triple-GR10924 duplicate | AACGCTTAGATGTGAC | 94.1 |
| VP2_5'UTR (LC178565) | GGCTATTAAAGGCTCA | NA |
| rSA11/triple-GR10924 | GGCTCA | 37.5 |
| rSA11/triple-GR10924 duplicate | GGCTATTAAAGGCTCA | 100.0 |
| VP2_3'UTR (LC178565) | ACGCCAACCCCATTGTGGAGATATGACC | NA |
| rSA11/triple-GR10924 | ACGCCAACCCCATTGTGGAGATA | 82.1 |
| rSA11/triple-GR10924 duplicate | ACGCCAACCCCATTGTGGAGATATG | 89.3 |
| VP3_5'UTR (LC178566) | GGCTATTAAAGCAGTACCAGTAGTGTGTTTTACCTCTGATGGTGTAAAC | NA |
| rSA11/triple-GR10924 | AAGCAGTACCAGTAGTGTGTTTTACCTCTGATGGTGTAAAC | 83.7 |
| rSA11/triple-GR10924 duplicate | AGCAGTACCAGTAGTGTGTTTTACCTCTGATGGTGTAAAC | 81.6 |
| VP3_3'UTR (LC178566) | GCTAAAAACTTAACACACTAGTCATGATGTGACC | NA |
| rSA11/triple-GR10924 | GCTAAAAACTTAACACACTAGTCATGATGTG | 91.2 |
| rSA11/triple-GR10924 duplicate | GCTAAAAACTTAACACACTAGTCATGATGTGACC | 100.0 |
| VP4_5'UTR (FJ183356) | GGCTATAAA | NA |
| rSA11/triple-GR10924 | AAA | 33.3 |
| rSA11/triple-GR10924 duplicate | – | 0.0 |
| VP4_3'UTR (FJ183356) | GAGCTCTATAGAGGATGTGACC | NA |
| rSA11/triple-GR10924 | GAGCTCTATAGAGGATGTGAC | 95.5 |
| rSA11/triple-GR10924 duplicate | GAGCTCTATAGAGGATGTGACC | 100.0 |
| VP6_5'UTR (FJ183358) | GGCTTTAAAACGAAGTCTTCAAC | NA |
| rSA11/triple-GR10924 | CGAAGTCTTCAAC | 56.5 |
| rSA11/triple-GR10924 duplicate | GGCTTTAAAACGAAGTCTTCAAC | 100.0 |
| VP6_3'UTR (FJ183358) | GGACCAAGCTAACCACTTGGTATCCAACTTTGATGAGTATGTAGCTACGTCAAGCTGTTTGAACTCTGTAAGTAAGGATGCGCTTACGTATTCGCTACACAGAGTAATCACTCAGATGACGTAGTGAGAGGATGTGACC | NA |
| rSA11/triple-GR10924 | GGACCAAGCTAACCACTTGGTATCCAACTTTGATGAGTATGTAGCTACGTCAAGCTGTTTGAACTCTGTAAGTAAGGATGCGCTTACGTATTCGCTACACAGAGTAATCACTCAGATGACGTAGTGAGAGGATGTGAC | 99.3 |
| rSA11/triple-GR10924 duplicate | GGACCAAGCTAACCACTTGGTATCCAACTTTGATGAGTATGTAGCTACGTCAAGCTGTTTGAACTCTGTAAGTAAGGATGCGCTTACGTATTCGCTACACAGAGTAATCACTCAGATGACGTAGTGAGAGGATGTGACC | 100.0 |
| VP7_5'UTR (FJ183360) | GGCTTTAAAAGAGAGAATTTCCGTTTGGCTAGCGGTTAGCTCCTTTTA | NA |
| rSA11/triple-GR10924 | AGAGAATTTCCGTTTGGCTAGCGGTTAGCTCCTTTTA | 77.1 |
| rSA11/triple-GR10924 duplicate | AGAGAATTTCCGTTTGGCTAGCGGTTAGCTCCTTTTA | 77.1 |
| VP7_3'UTR (FJ183360) | TATATCTTAGGTTAGAATTGGTCGATGTGACC | NA |
| rSA11/triple-GR10924 | TATATCTTAGGTTAGAATTGGTC | 71.9 |
| rSA11/triple-GR10924 duplicate | TATATCTTAGGTTAGAATTGGTCGATGTGA | 93.8 |

**Table S4**. UTRs of the genome segments encoding non-structural viral proteins from rSA11/triple-GR10924 and rSA11/triple-GR10924 duplicate. Accession numbers of reference sequences are shown in brackets. % UTR = percentage of the UTR identified by sequencing in relation to the reference sequence; NA = Not applicable. The exchanged nucleotide in NSP4 is underlined and in bold.

| Segment | Sequence 5' -> 3' | % UTR |
|---|---|---|
| NSP1_5'UTR (LC178570) | GGCTTTTTTTTGAAAAGTCTTGTGTTAGCC | NA |
| rSA11/triple-GR10924 | GAAAAGTCTTGTGTTAGCC | 63,3 |
| rSA11/triple-GR10924 duplicate | TTTTTTTTTGAAAAGTCTTGTGTTAGCC | 90,0 |
| NSP1_3'UTR (LC178570) | AATTATGTCACTATCTAATTATACAGTATTTAGCCATCACAAGACCGTCCAG ACTAGAGTAGCGCCTAGCTGGCAAAATACTGTGAACC | NA |
| rSA11/triple-GR10924 | AATTATGTCACTATCTAATTATACAGTATTTAGCCATCACAAGACCGTCCAG ACTAGAGTAGCGCCTAGCTGGCAAAATACTGTG | 95.5 |
| rSA11/triple-GR10924 duplicate | AATTATGTCACTATCTAATTATACAGTATTTAGCCATCACAAGACCGTCCAG ACTAGAGTAGCGCCTAGCTGGCAAAATACTGTGAAC | 98.9 |
| NSP2_5'UTR (LC178571) | GGCTTTTAAAGCGTCTCAGTCGCCGTTTGAGCCTTGCGGTGTAGCC | NA |
| rSA11/triple-GR10924 | AGCGTCTCAGTCGCCGTTTGAGCCTTGCGGTGTAGCC | 80,4 |
| rSA11/triple-GR10924 duplicate | CGTCTCAGTCGCCGTTTGAGCCTTGCGGTGTAGCC | 76,1 |
| NSP2_3'UTR (LC178571) | TTCGCTATCAATTTGAGGATGATGATGGCTTAGCAAGAATAGAAAGCGCTTA TGTGACC | NA |
| rSA11/triple-GR10924 | TTCGCTATCAATTTGAGGATGATGATGGCTTAGCAAGAATAGAAAGCGCTTA TGTGACC | 100.0 |
| rSA11/triple-GR10924 duplicate | TTCGCTATCAATTTGAGGATGATGATGGCTTAGCAAGAATAGAAAGCGCTTA TGTGACC | 100.0 |
| NSP3_5'UTR (LC178572) | GGCATTTAATGCTTTTCAGTGGTTG | NA |
| rSA11/triple-GR10924 | AATGCTTTTCAGTGGTTG | 72.0 |
| rSA11/triple-GR10924 duplicate | GGCATTTAATGCTTTTCAGTGGTTG | 100.0 |
| NSP3_3'UTR (LC178572) | CCATTTTGATACATGTTGAACAATCAAATACAGTGTTAGTATGTTGTCATCT ATGCATAACCCTCTATGAGCACAATAGTTAAAAGCTAACACTGTCAAAAACC TAAATGGCTATAGGGGCGTTATGTGGCC | NA |
| rSA11/triple-GR10924 | CCATTTTGATACATGTTGAACAATCAAATACAGTGTTAGTATGTTGTCATCT ATGCATAACCCTCTATGAGCACAATAGTTAAAAGCTAACACTGTCAAAAACC TAAATGGCTATAGGGGCGTTATGT | 97.0 |
| rSA11/triple-GR10924 duplicate | CCATTTTGATACATGTTGAACAATCAAATACAGTGTTAGTATGTTGTCATCT ATGCATAACCCTCTATGAGCACAATAGTTAAAAGCTAACACTGTCAAAAACC TAAATGGCTATAGGGGCGTTATG | 96.2 |
| NSP4_5'UTR (LC178573) | GGCTTTTAAAAGTTCTGTTCCGAGAGAGCGCGTGCGGAAAG | NA |
| rSA11/triple-GR10924 | CTGTTCCGAGAGAGCGCGTGCGGAAAG | 65.9 |
| rSA11/triple-GR10924 duplicate | AGTTCTGTTCCGAGAGAGCGCGTGCGGAAAG | 75.6 |
| NSP4_3'UTR (LC178573) | GAGGTTGAGCTGCCGTCGACTGTCCTCGGAAGCGGCGGAGTTCTTTACAGTA AGCACCATCGGACCTGATGGCTGACTGAGAAGCCACAGTCAGCCATATCGCG TGTGGCTCAAGCCTTAATCCCGTTTAACCAATCCGGTCAGCACCGGACGTTA ATGGAAGGAACGGTCTTAATGTGACC | NA |
| rSA11/triple-GR10924 | GAGGTTGAGCTGCCGTCGACTGTCCTCGGAAGCGGCGGAGTTCTTTACAGTA AGCACCATCGGACCTGATGGCTGACTGAGAAGCCACAGTCAGCCATATCGCG TGTGGCTCAAGCCTTAATCCCGTTTAACCAATCCGGTCAGCACCGGACGTTA ATGGAAGGAACGG | 92.9 |
| rSA11/triple-GR10924 duplicate | GAGGTTGAGCTGCCGTCGACTGTCCTCGGAAGCGGCGGAGTTCTTTACAGTA AGCACCATCGGACCTGATGGCTGACTGAGAAGCCACAGTCAGCCATATCGCG TGTGGCTCAAGCCTTAATCCCGTTTAACCAA**c**CGGTCAGCACCGGACGTTAA TGGAAGGAAC | 91.2 |
| NSP5_5'UTR (LC178574) | GGCTTTTAAAGCGCTACAGTG | NA |
| rSA11/triple-GR10924 | GCGCTACAGTG | 56.9 |
| rSA11/triple-GR10924 duplicate | GCGCTACAGTG | 75.6 |
| NSP5_3'UTR (LC178574) | GTCTGACCTGGGAACACACTAGGGAGCTCCCCACTCCCGTTTTGTGACC | NA |
| rSA11/triple-GR10924 | GTCTGACCTGGGAACACACTAGGGAGCTCCCCACTCCCGTT | 83.7 |
| rSA11/triple-GR10924 duplicate | GTCTGACCTGGGAACACACTAGGGAGCTCCCCACTC | 73.5 |