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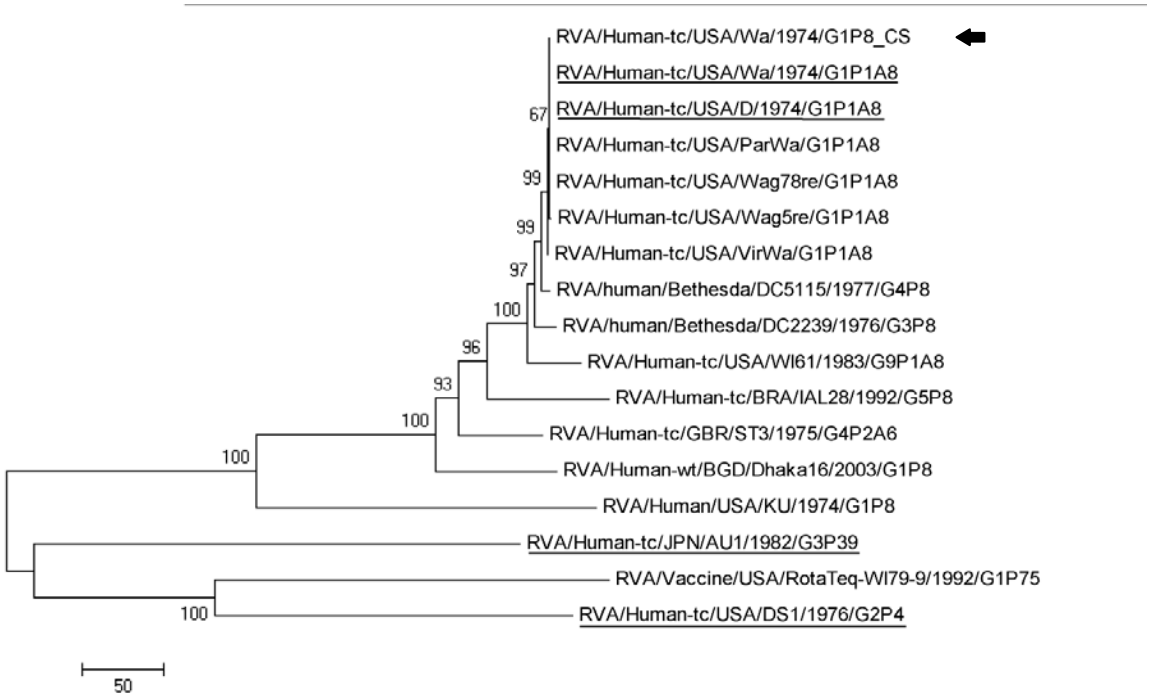
APPENDIX A

PHYLOGENETIC ANALYSIS

Genome segment 1 (VP1)



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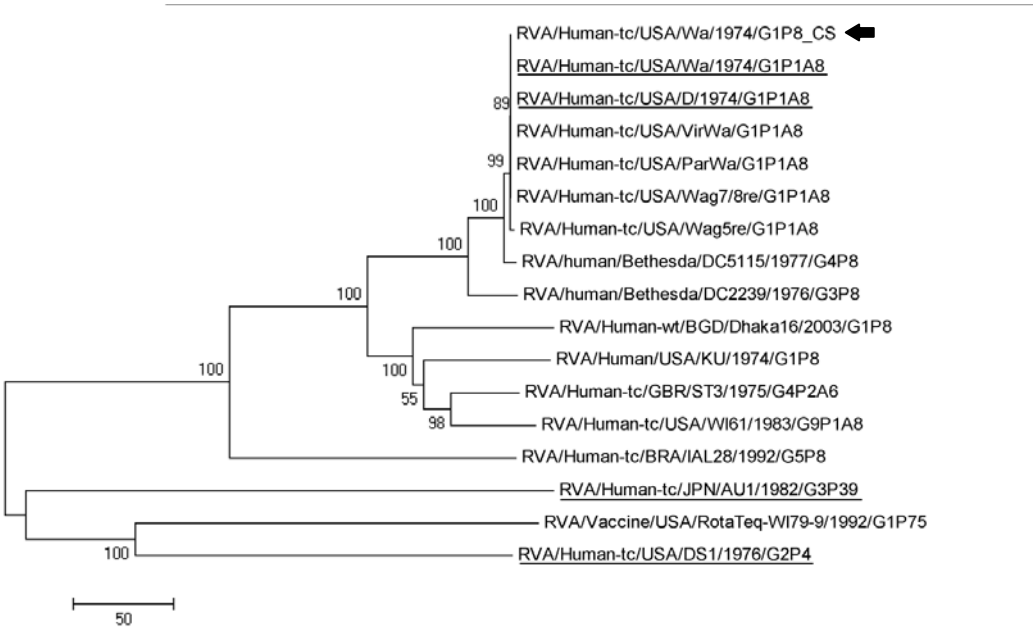

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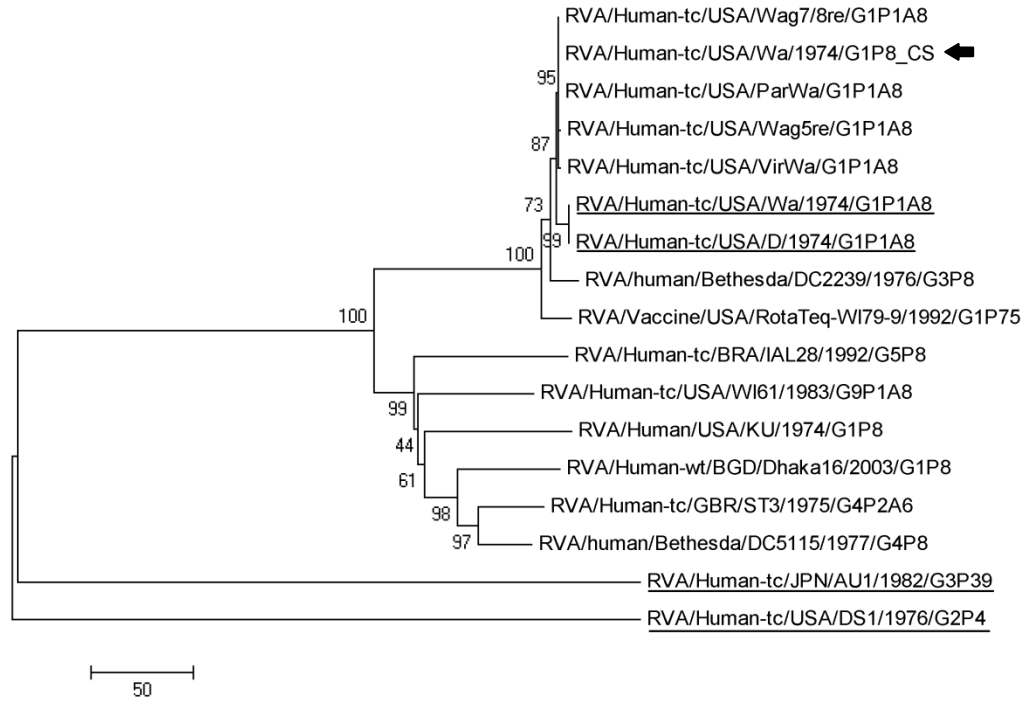

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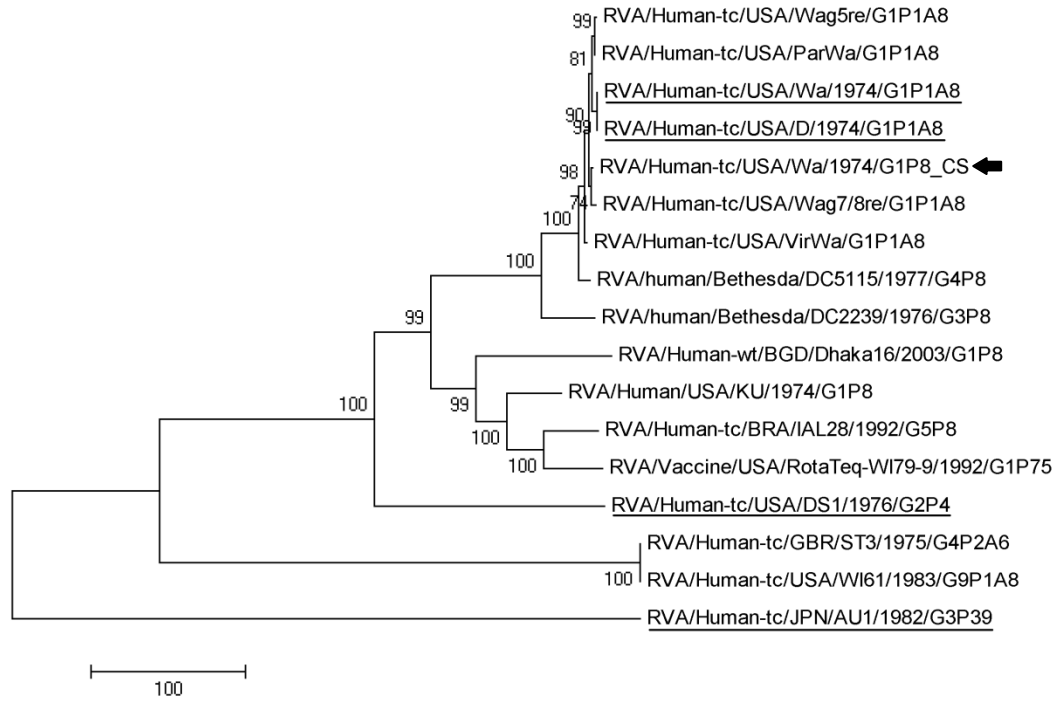
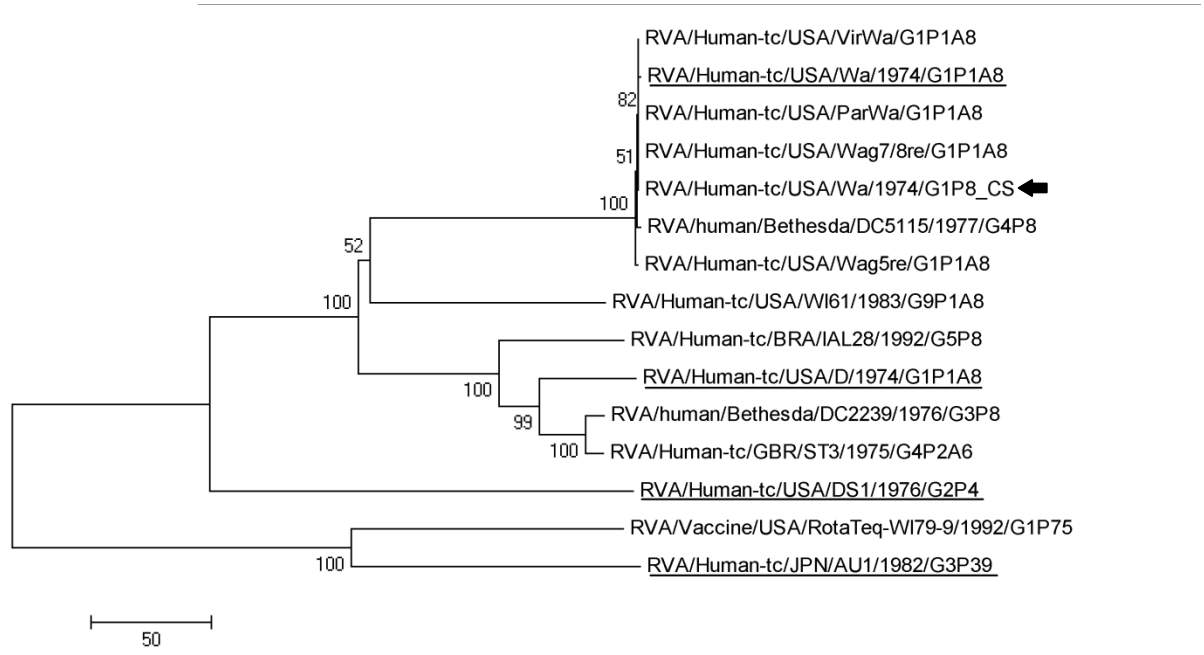


Genome segment 5 (NSP1)



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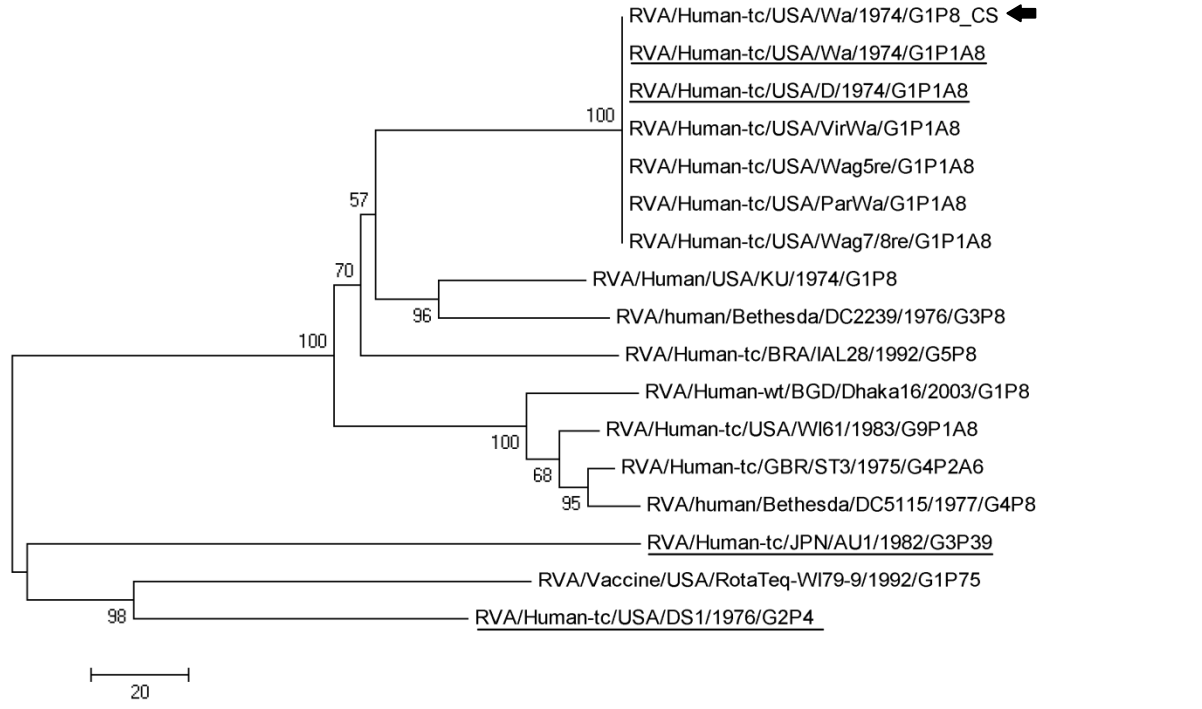

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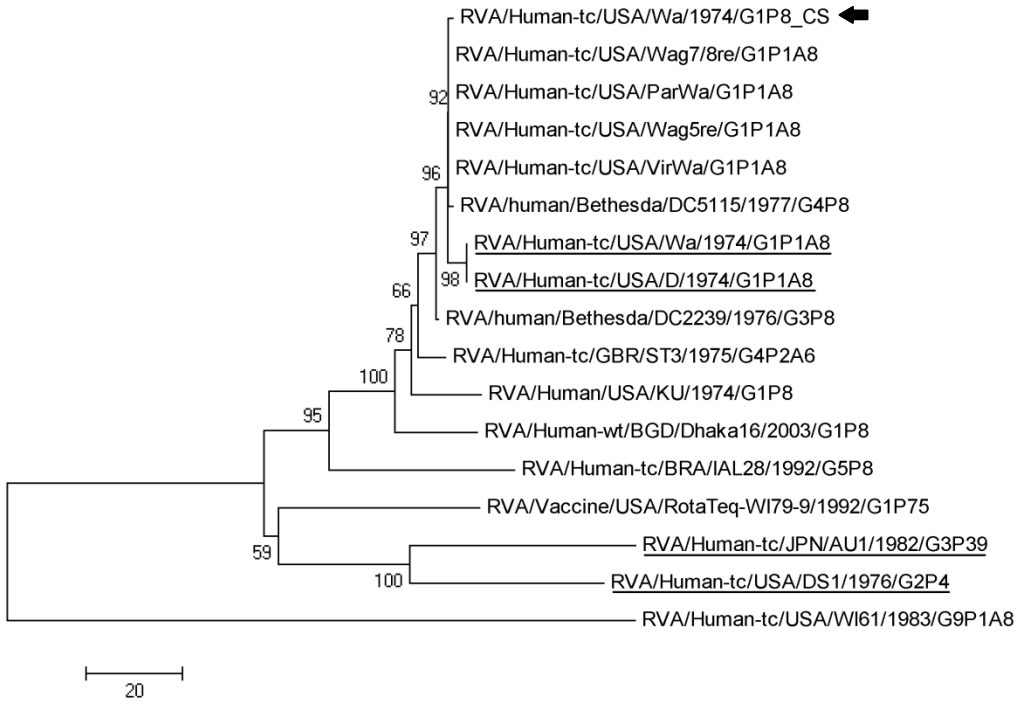
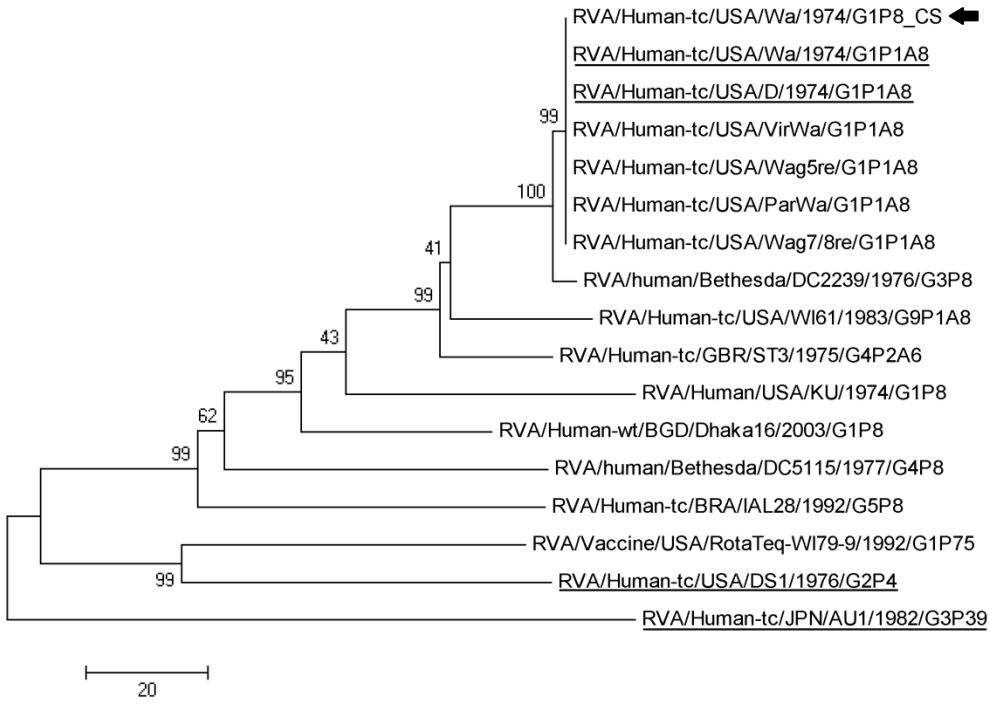


Genome segment 8 (NSP2)



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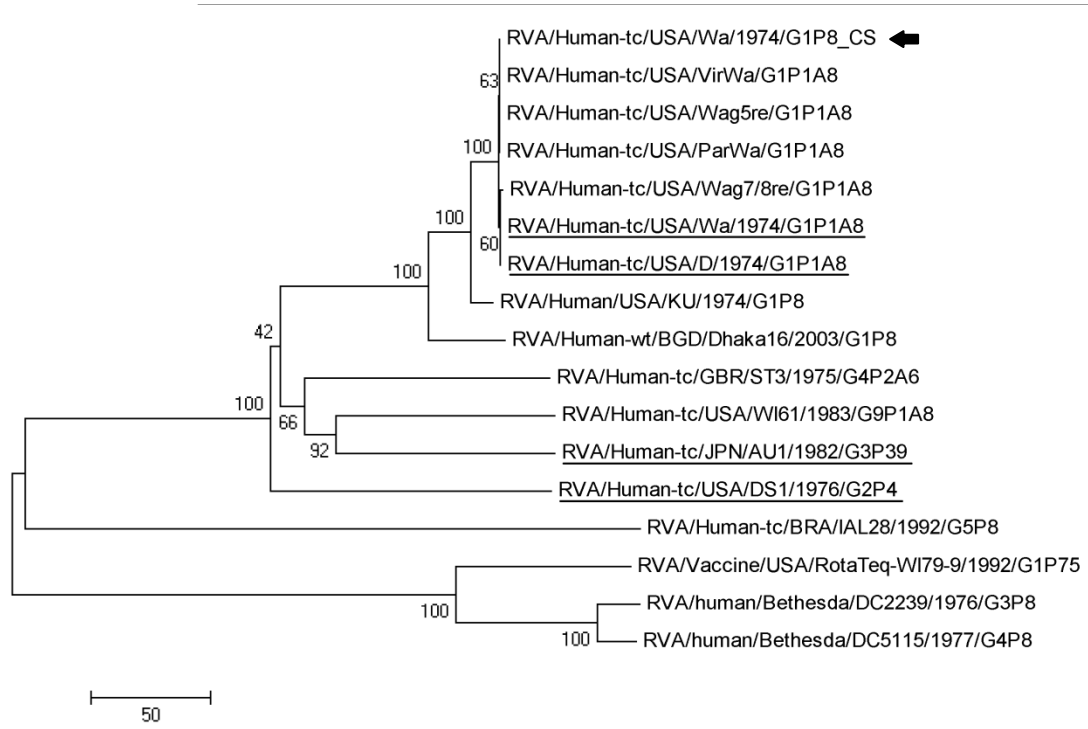

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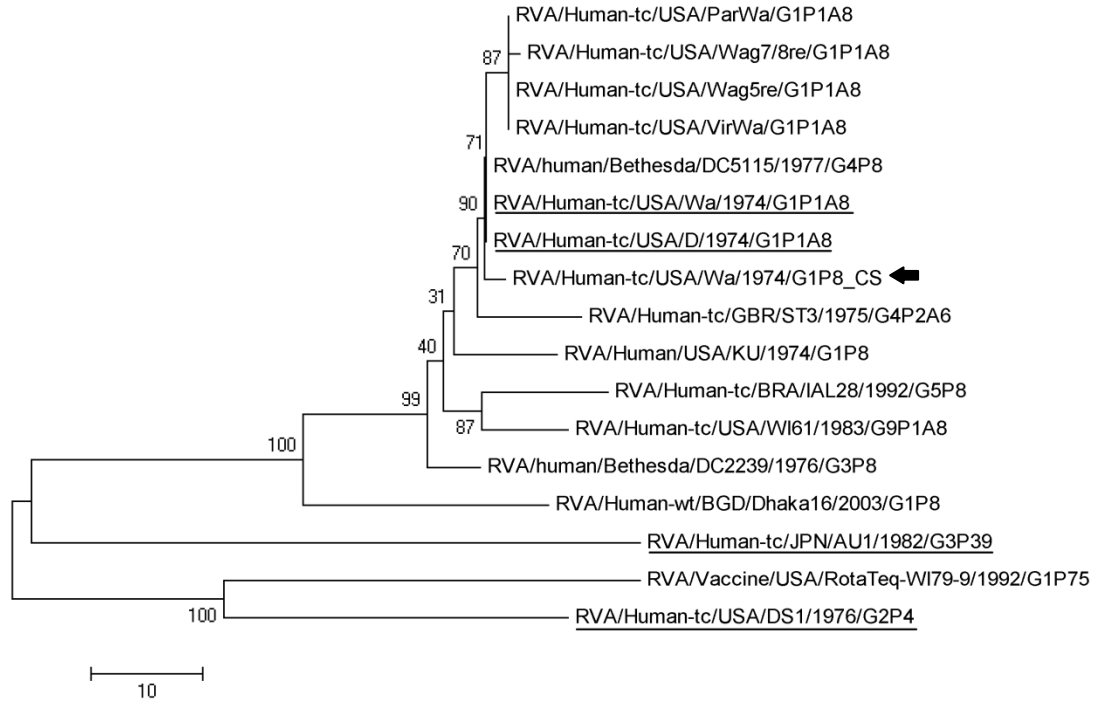
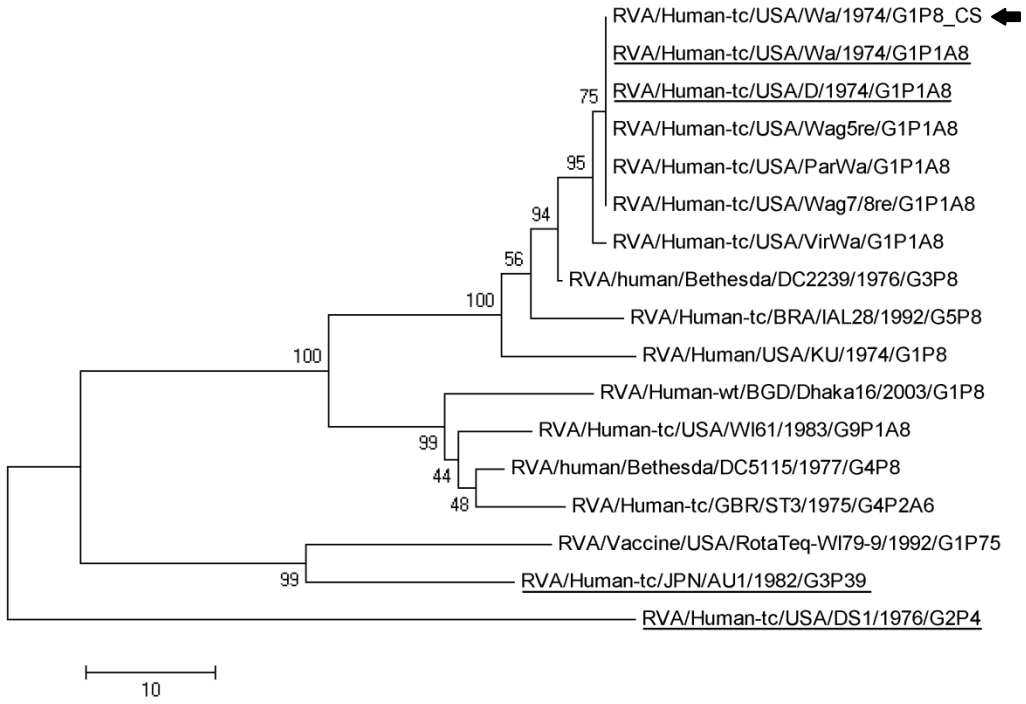


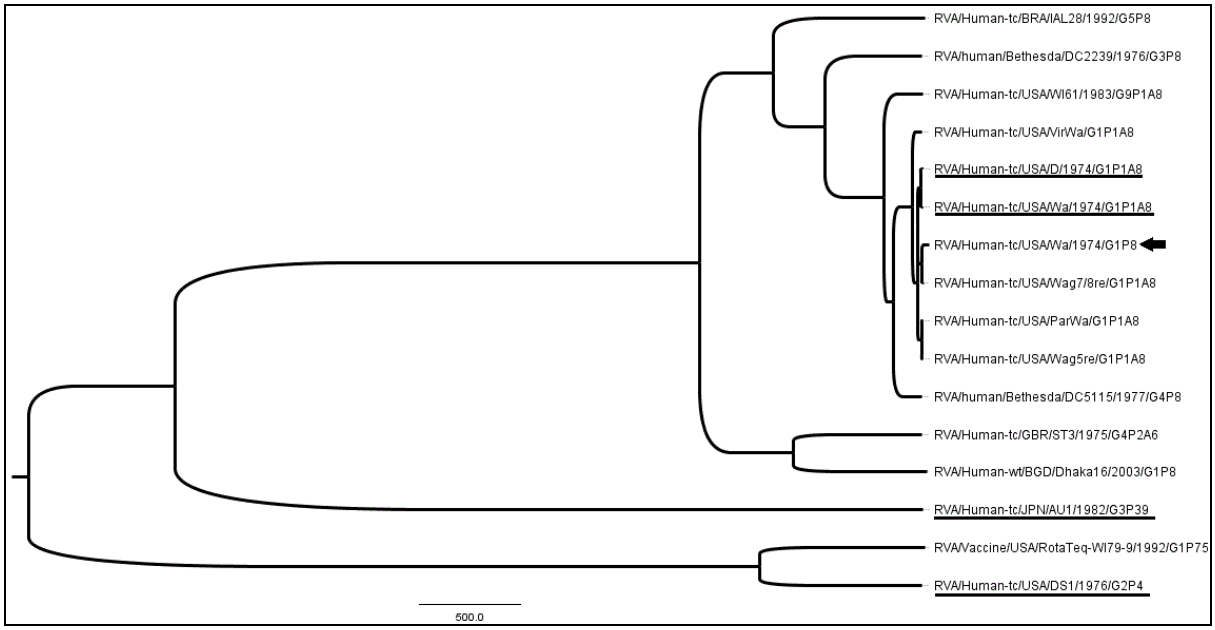
Genome segment (NSP5/6)



Supplementary Figure A.1 : ***Nucleotide sequence based phylogenetic trees of the genome segments encoding structural (VP1–VP4, VP6, VP7) and non-structural (NSP1–NSP5/6) proteins.*** *The evolutionary history was inferred using the Neighbour-Joining method and the evolutionary distances were computed using the number of differences method and are in the units of the number of base differences per sequence. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. The arrows indicate the sequenced rotavirus Wa strain. The prototype strains are underlined. Scale bars are proportional to the phylogenetic distance.*

MCC MOLECULAR CLOCK ANALYSIS

Genome segment 1 (VP1)



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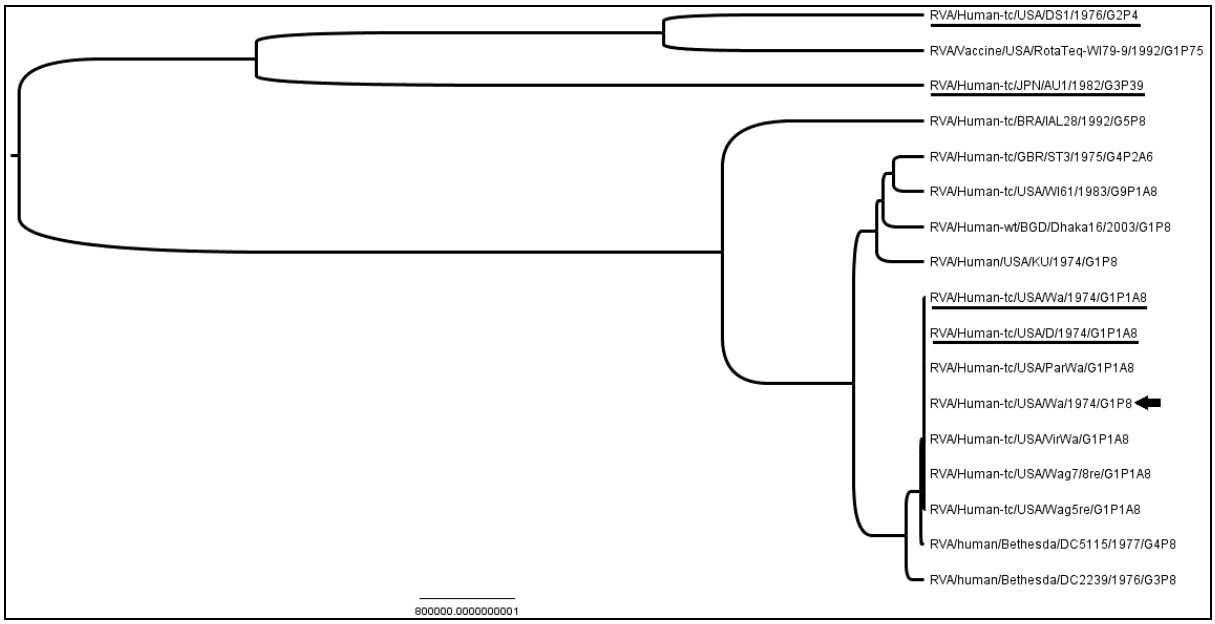

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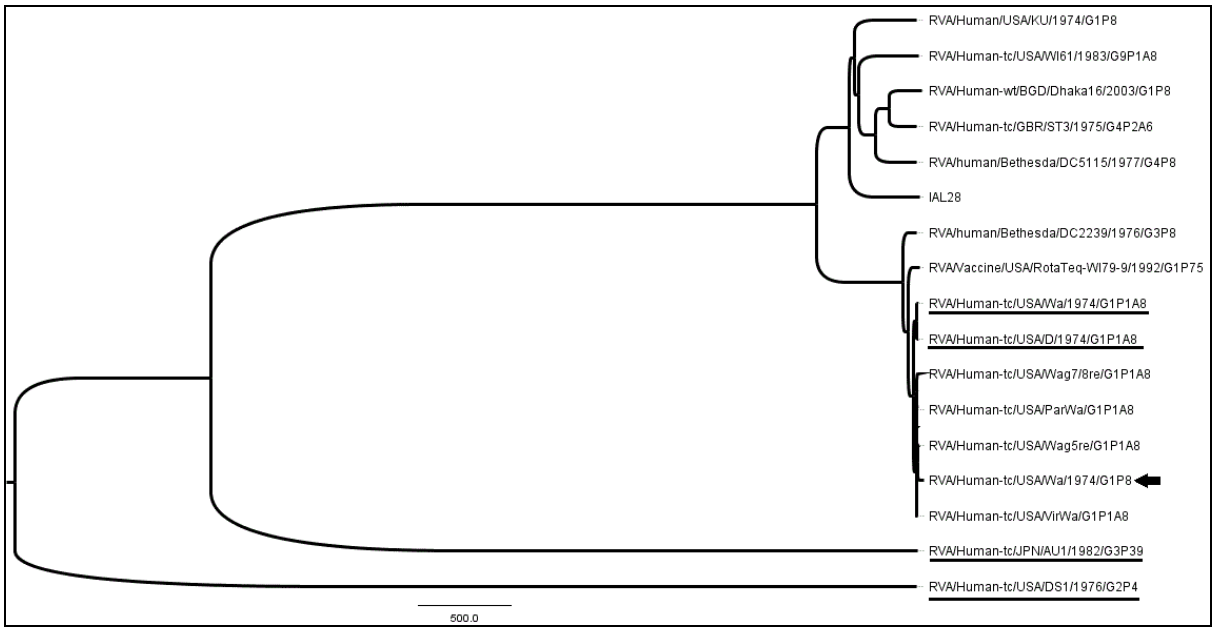

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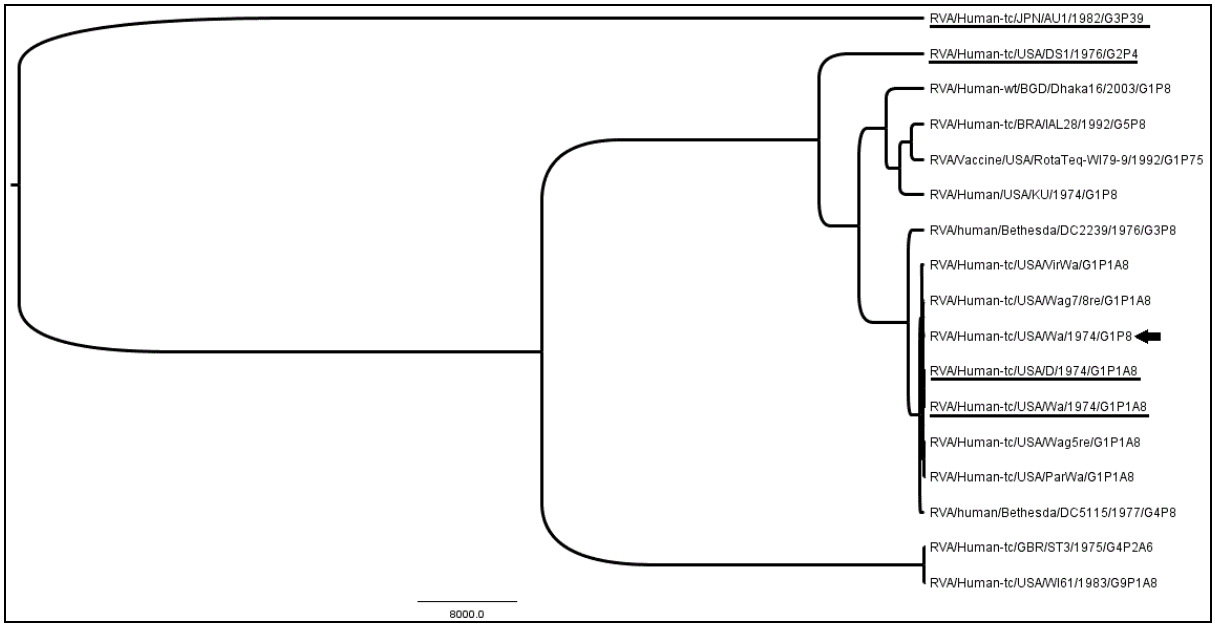

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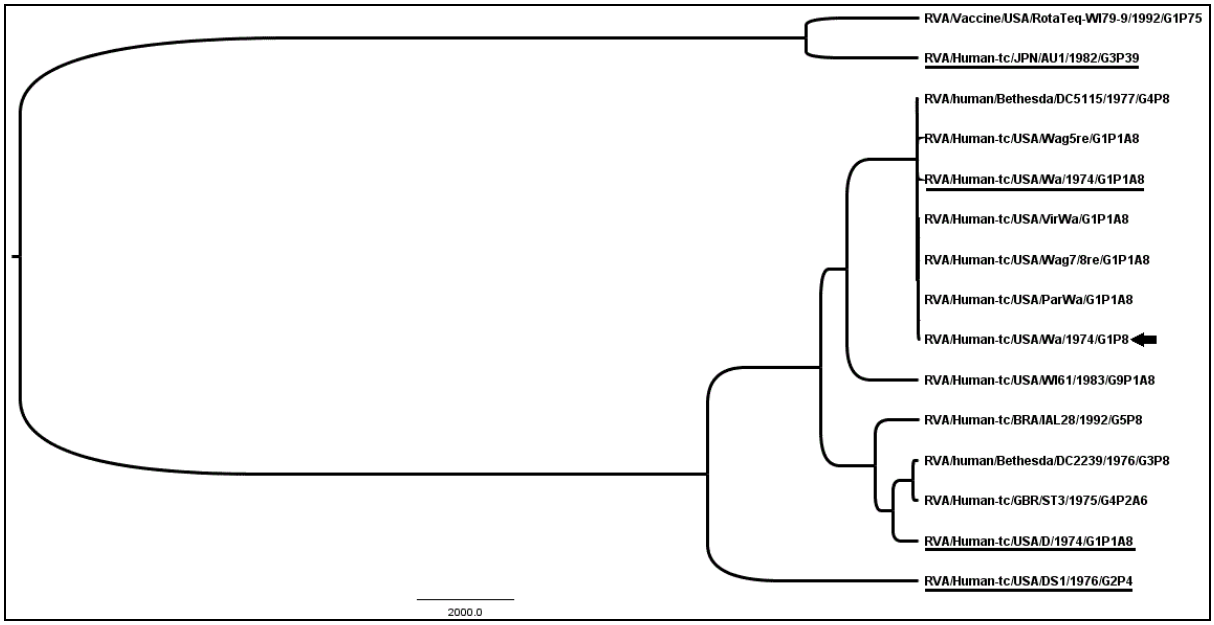
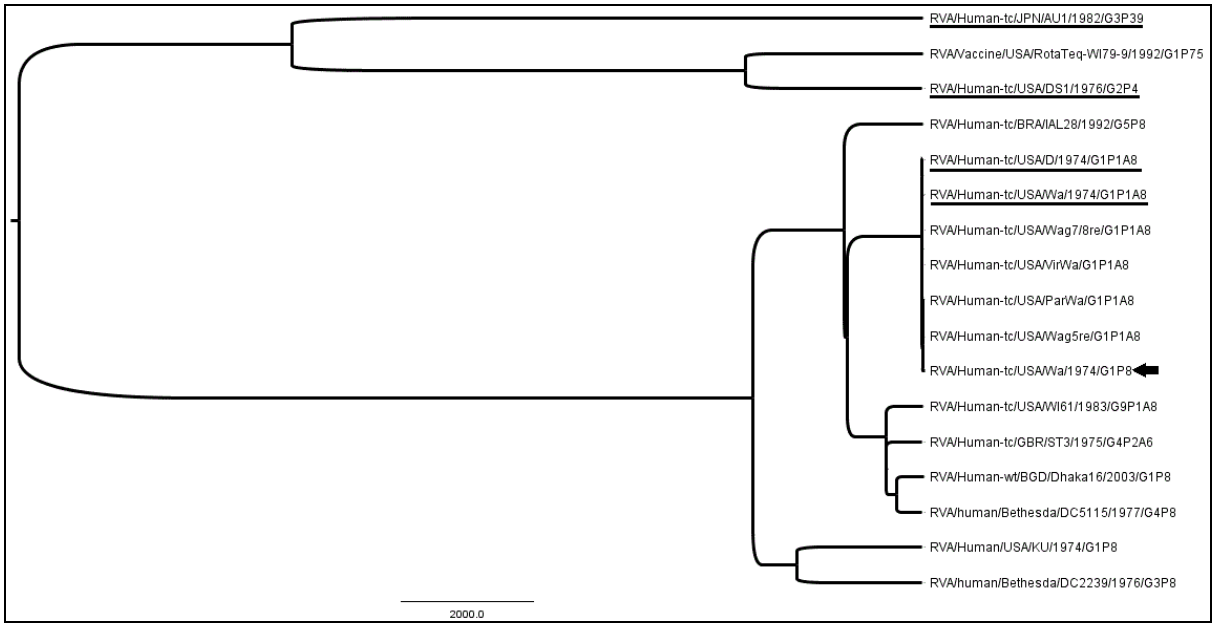
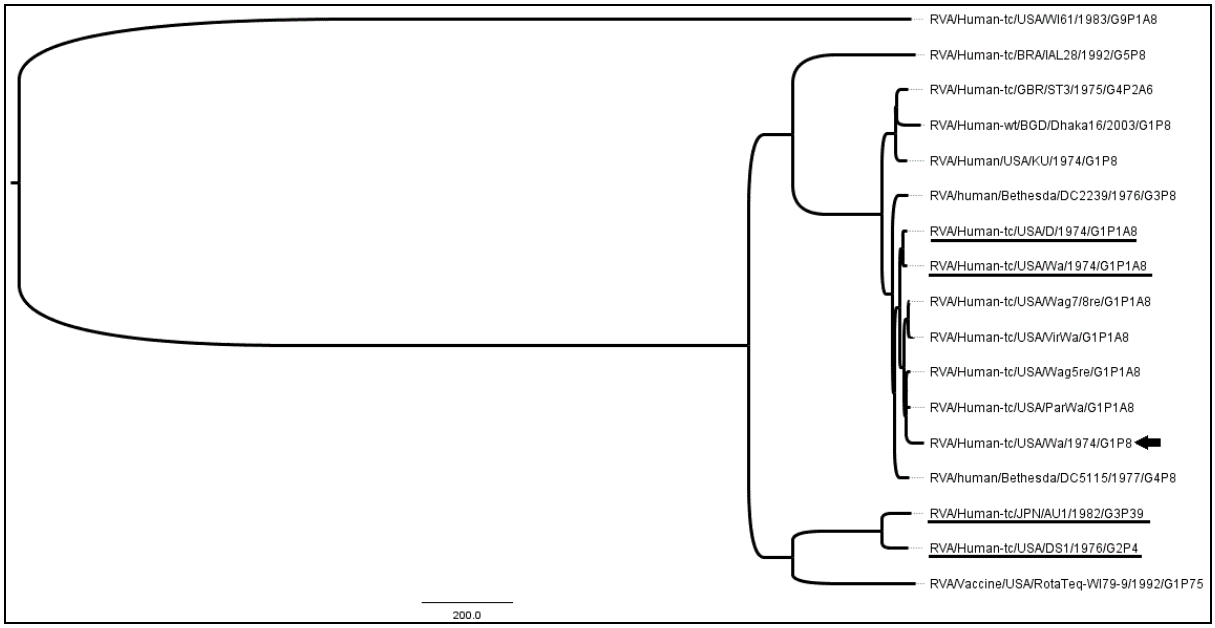


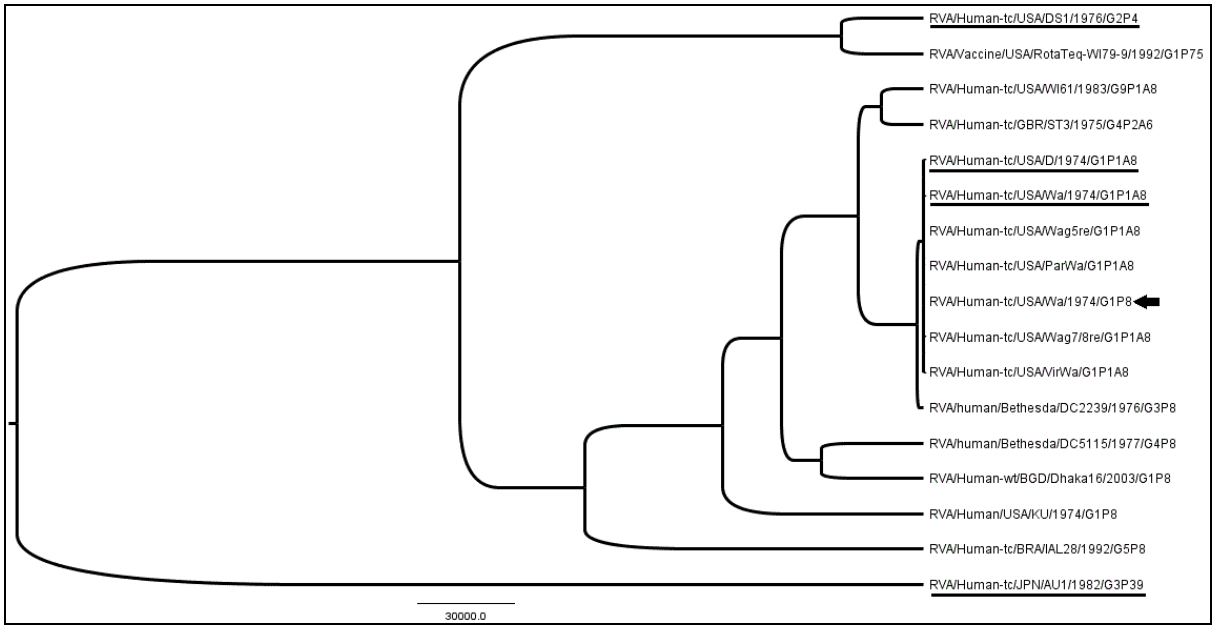
Genome segment 6 (VP6)



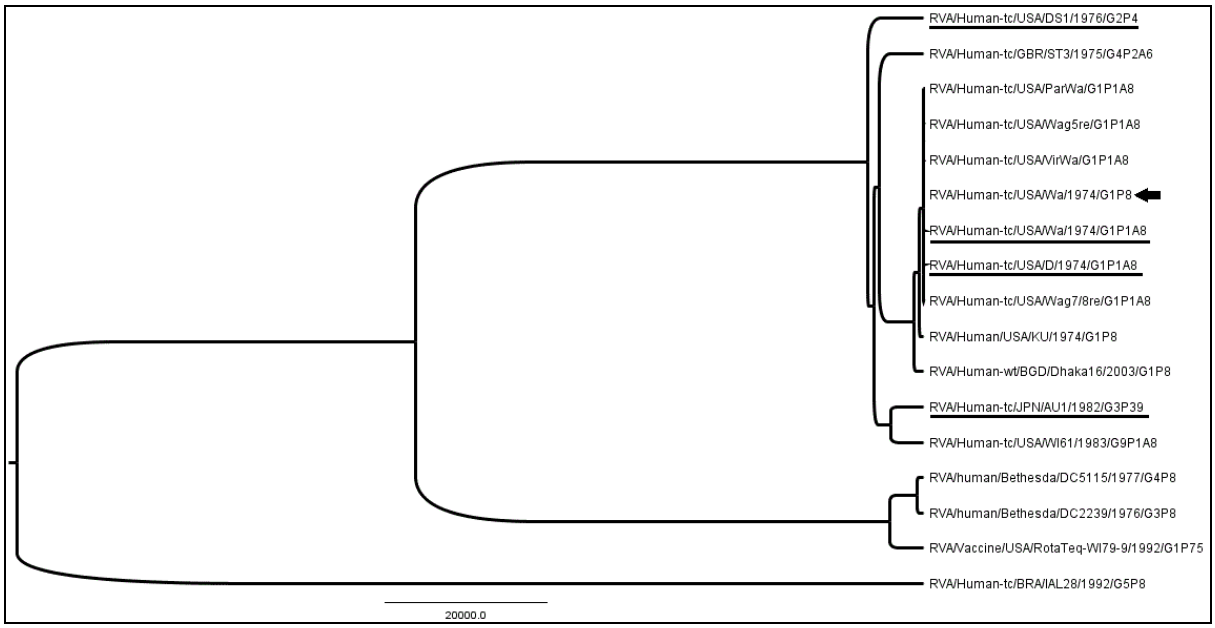
Genome segment 7 (NSP3)



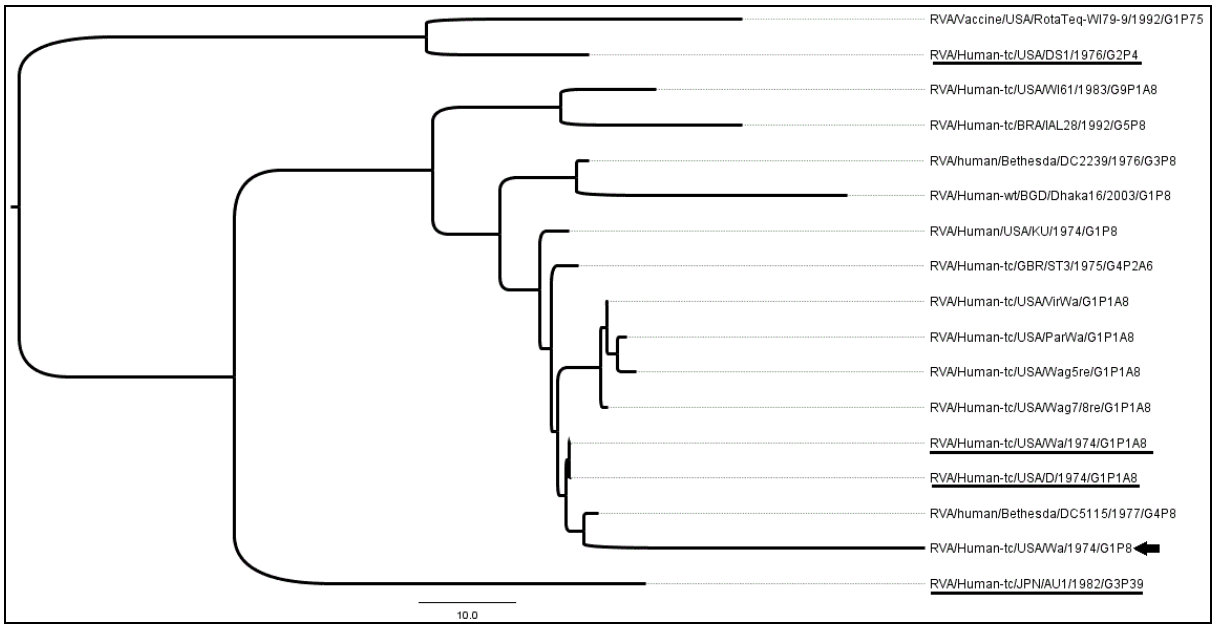
Genome segment 8 (NSP2)



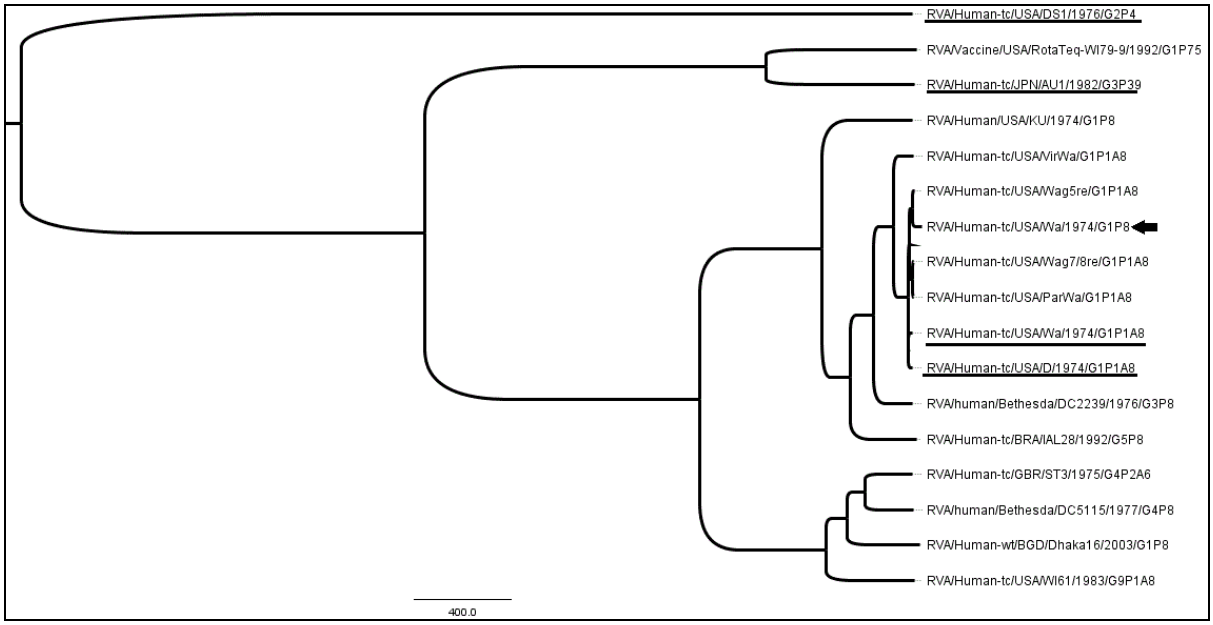
Genome segment 9 (VP7)



Genome segment 10 (NSP4)



Genome segment (NSP5/6)



Supplementary Figure A.2: ***Maximum clade credibility (MCC) trees of all 11 genome segments of the 17 rotavirus genome sequences analysed using the Bayesian MCMC framework.*** *The sequenced consensus strain is indicated with an arrow and the reference strains are underlined.*

Supplementary Table A.1:

Pairwise comparisons of the number of nucleotide and amino acid differences of the closest related rotavirus strains in GenBank in comparison to the rotavirus WaCS determined in this study

		WaCS GS1 (VP1)	WaCS GS2 (VP2)	WaCS GS3 (VP3)	WaCS GS4 (VP4)	WaCS GS5 (NSP1)	WaCS GS6 (VP6)	WaCS GS7 (NSP3)	WaCS GS8 (NSP2)	WaCS GS9 (VP7)	WaCS GS10 (NSP4)	WaCS GS11 (NSP5/6)
RVA/Wa/G1P1A8 (Ref.)	No. of nucleotide changes	1	0	7	8	4	1	15	0	2	2	1
	No. of amino acid changes	0	0	2	7	3	0	14	0	1	2	0
RVA/VirWa/G1P1A8	No. of nucleotide changes	0	0	1	7	1	0	1	0	1	4	2
	No. of amino acid changes	0	0	1	6	0	0	1	0	1	3	2
RVA/Wag7/8re/G1P1A8	No. of nucleotide changes	0	0	1	4	2	0	> 926 [b]	> 927 [c]	4	5	0
	No. of amino acid changes	0	0	0	4	1	0	1	0	1	4	0
RVA/ParWa/G1P1A8	No. of nucleotide changes	0	0	0	7	2	0	1	0	1	4	0
	No. of amino acid changes	0	0	0	7	1	0	1	0	1	2	0
RVA /Wa g5re /G1P	No. of nucleotide changes	1	2	2	8	> 972 [a]	0	1	0	1	4	0

	No. of amino acid changes	1	1	0	8	n/a	0	1	0	1	2	0
RVA/IAL28/ G5P8	No. of nucleotide changes	112	280	186	210	227	106	56	116	541	20	13
	No. of amino acid changes	17	20	32	42	79	6	27	20	70	7	4/1
RVA/ST3/G 4P2A6	No. of nucleotide changes	107	147	175	589	218	116	11	39	217	12	40
	No. of amino acid changes	19	19	31	174	73	8	4	10	77	5	13/4
RVA/WI61/G 9P1A8	No. of nucleotide changes	46	156	167	589	209	111	277	42	226	17	37
	No. of amino acid changes	5	23	33	241	67	10	11	13	68	7	12/3
RVA/RotaTeq-WI79-9/G1P75	No. of nucleotide changes	133	519	22	215	515	229	82	173	7	103	78
	No. of amino acid changes	105	65	6	40	205	32	40	35	5	26	24/na
RVA/DC223 9/G3P8	No. of nucleotide changes	22	44	17	66	129	100	3	6	535	12	4
	No. of amino acid changes	8	7	3	11	74	5	2	2	57	3	2/0
RVA /DC5 115/ G4P	No. of nucleotide changes	10	9	168	15	2	124	2	111	532	2	35

	No. of amino acid changes	2	1	25	6	0	9	2	13	77	2	11/3
RVA/Dhaka 16/G1P8	No. of nucleotide changes	144	165	181	221	176	123	24	72	73	41	42
	No. of amino acid changes	13	20	32	42	80	9	6	12	19	8	13/na
RVA/D/G1P 1A8	No. of nucleotide changes	4	2	7	20	234	0	4	0	6	4	0
	No. of amino acid changes	4	2	4	12	81	3	3	0	5	3	3/0
RVA/AU1/G 3P39	No. of nucleotide changes	652	534	570	791	516	257	103	196	224	98	75
	No. of amino acid changes	94	65	139	274	208	33	51	35	59	28	21
RVA/DS1/G 2P4	No. of nucleotide changes	684	508	579	287	353	215	96	174	235	97	94
	No. of amino acid changes	109	76	154	80	150	31	57	35	83	29	34/4

[a]Genome segment 5 of the RV variant Wag5re yields a non-functioning NSP1 due to a 972 bp insert

[b]Genome segment 7 of the RV variant VirWa, contains a 926 bp insert but the ORF is not influenced

[c]Genome segment 8 of the RV variant Wag7/8re contains a 927 bp insert but the ORF is not influenced

APPENDIX B

ORIGINAL ARTICLE

Wentzel, J.F., Yuan, L., Rao, S., van Dijk, A.A. and O'Neill, H.G. 2013. Consensus sequence determination and elucidation of the evolutionary history of a rotavirus Wa variant reveal a close relationship to various Wa variants derived from the original Wa strain. Infection, Genetics and Evolution. 20. 276-283

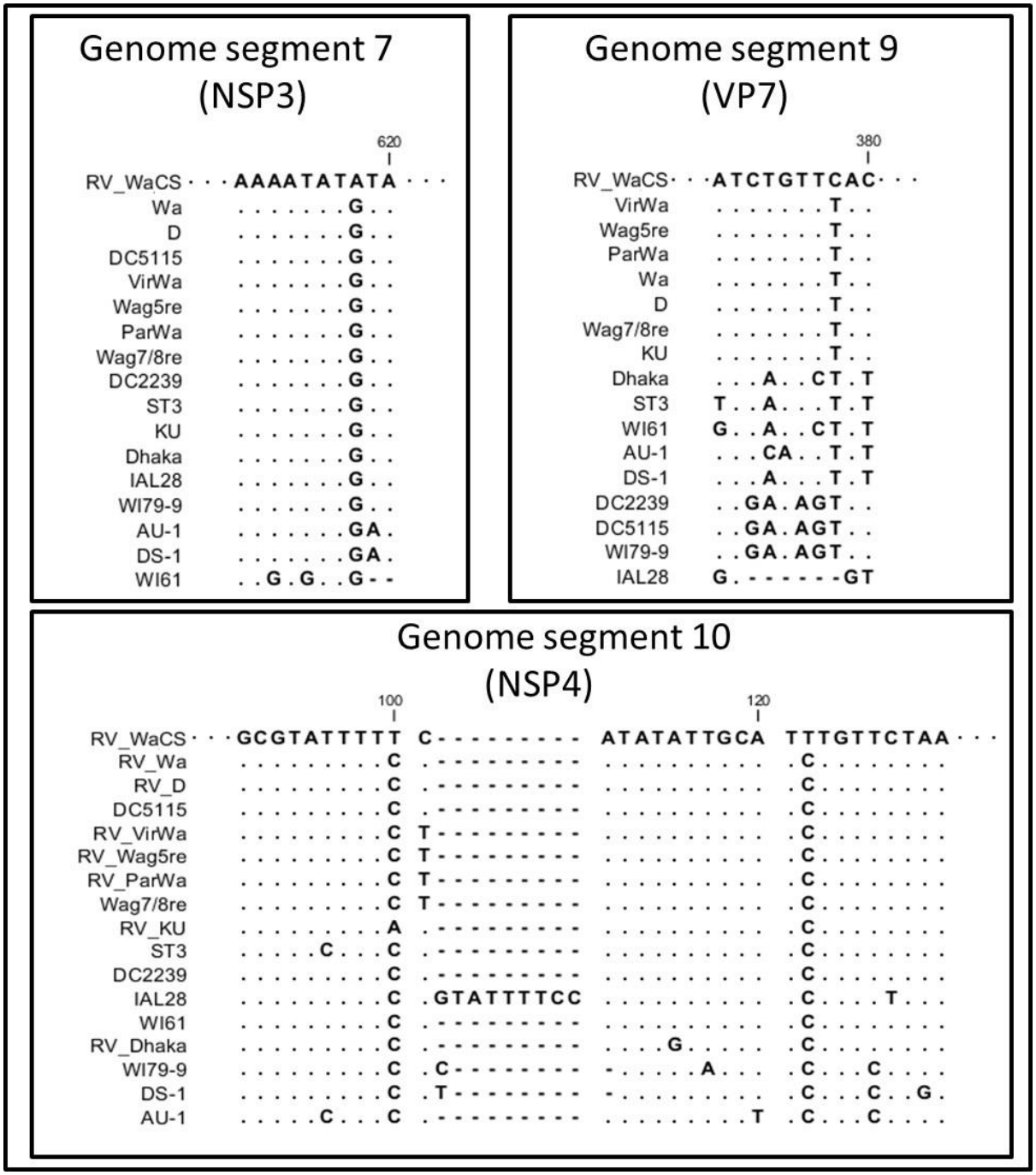
Link:

http://www.sciencedirect.com/science/article/pii/S1567134813003456

Article Supplementary Table 1: GenBank accession numbers of rotavirus strains used in phylogenetic analysis and pairwise comparisons

Type	Rotavirus strain	GenBank accession numbers of different rotavirus genome segments										
		GS1 (VP1)	**GS2 (VP2)**	**GS3 (VP3)**	**GS4 (VP4)**	**GS5 (NSP1)**	**GS6 (VP6)**	**GS7 (NSP3)**	**GS8 (NSP2)**	**GS9 (VP7)**	**GS10 (NSP4)**	**GS11 (NSP5/6)**
Wa-like	RVA/Human-tc/USA/**WaCS**/1974/G1P1A[8]	DQ490539	X14942	AY267335	L20877.1	L18943	K02086	X81434	L04534	M21843	AF093199	AF306494
	RVA/Human-tc/USA/**D**/1974/G1P1A[8]	EF583021	EF583022	EF583023	EF672570	EF672571	EF583024	EF672572	EF672573	EF672574	EF672575	EF672576
	VirWa G1P[8]	FJ423113	FJ423114	FJ423115	FJ423116	FJ423117	FJ423118	FJ423119	FJ423120	FJ423121	FJ423122	FJ423123
	Wag7/8re G1P[8]	FJ423135	FJ423136	FJ423137	FJ423138	FJ423139	FJ423140	FJ423141 .	FJ423142	FJ423143	FJ423144	FJ423145
	ParWa G1P[8]	FJ423124	FJ423125	FJ423126	FJ423127	FJ423128	FJ423129	FJ423130	FJ423131	FJ423132	FJ423133	FJ423134
	Wag5re G1P[8]	FJ423146	FJ423147	FJ423148	FJ423149	FJ423156	FJ423150	FJ423151	FJ423152	FJ423153	FJ423154	FJ423155
	RVA human/Bethesda/**DC5115**/1977/G4P[8]	HM773942	HM773943	HM773944	HM773945	HM773946	HM773947	HM773948	HM773949	HM773950	HM773951	HM773952
	RVA human/Bethesda/**DC2239**/1976/G3P[8]	FJ947859	FJ947860	FJ947861	FJ947862	FJ947863	FJ947864	FJ947865	FJ947866	FJ947867	FJ947868	FJ947869
	RVA/Human-wt/BGD/**Dhaka16**/2003/G1P[8]	DQ492669	DQ492670	DQ492671	DQ492672	DQ492675	DQ492673	DQ492677	DQ492676	DQ492674	DQ492678	DQ492679
	RVA/Human-tc/BRA/**IAL28**/1992/G5P[8]	EF583029	EF583030	EF583031	EF672584	EF672585	EF583032	EF672586	EF672587	EF672588	EF672589	EF672590
	RVA/Vaccine/USA/**RotaTeq-WI79-9**/1992/G1P7[5]	GU565052	GU565053	GU565054	GU565055	GU565058	GU565056	GU565060	GU565059	GU565057	GU565061	GU565062
	RVA/Human-tc/USA/**WI61**/1983/G9P1A[8]	EF583049	EF583050	EF583051	EF672619	EF672620	EF583052	EF672621	EF672622	EF672623	EF672624	EF672625
	RVA/Human-tc/GBR/**ST3**/1975/G4P2A[6]	EF583045	EF583046	EF583047	EF672612	EF672613	EF583048	EF672614	EF672615	EF672616	EF672617	EF672618
	KU G1P[8]	AB022765	AB022766	AB022767	AB222784	AB022769	AB022768	AB022771	AB022770	D16343	AB022772	AB022773
DS-1-like	RVA/Human-tc/USA/**DS-1**/1976/G2P1B[4]	HQ650116	HQ650117	HQ650118	HQ650119	HQ650120	HQ650121	HQ650122	HQ650123	HQ650124	HQ650125	HQ650126
AU-1-like	RVA/Human-tc/JPN/**AU-1**/1982/G3P3[9]	DQ490533	DQ490536	DQ490537	D10970	D45244	DQ490538	DQ490535	DQ490534	D86271	D89873	AB008656

Article Supplementary Table 2: ***Please see Supplementary Table A.1:*** Pairwise comparisons of the number of nucleotide and amino acid differences of the closest related rotavirus strains in GenBank in comparison to the rotavirus WaCS determined in this study

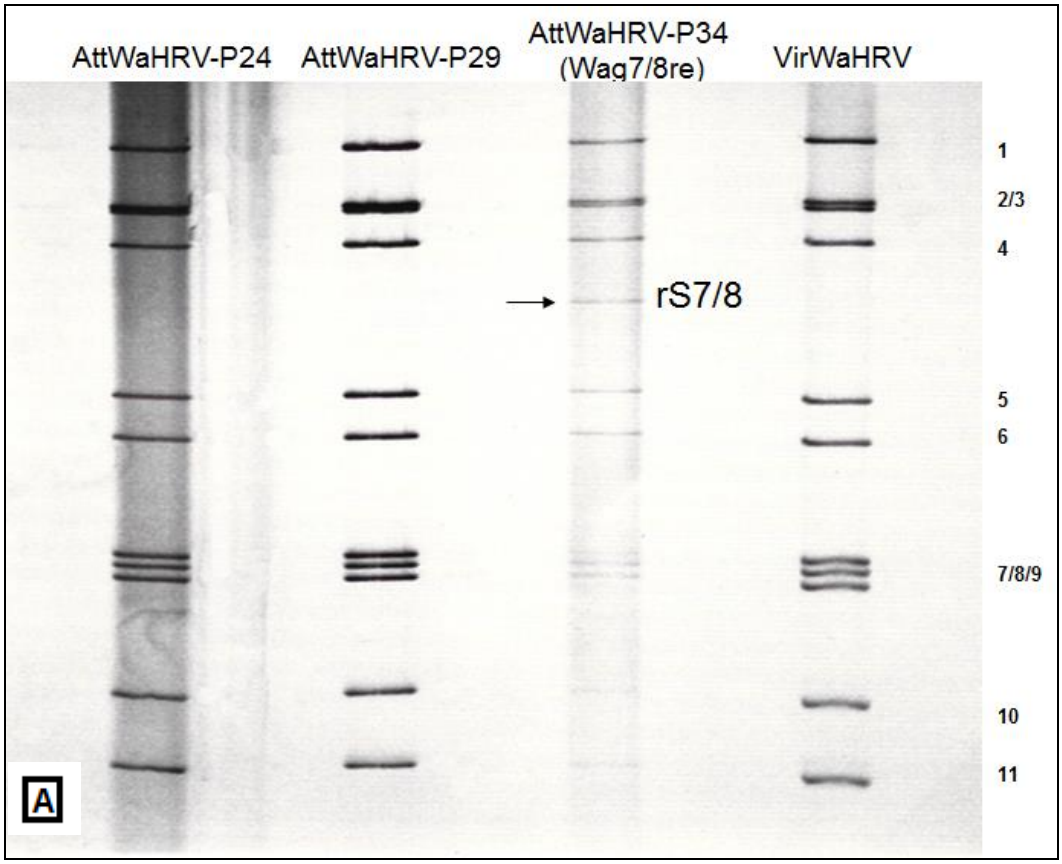


Article Supplementary Figure 1: Nucleotide alignments of WaCS, closely related Wa variants and rotavirus reference strains showing the novel nucleotide changes detected in genome segments 7, 9 and 10.

9

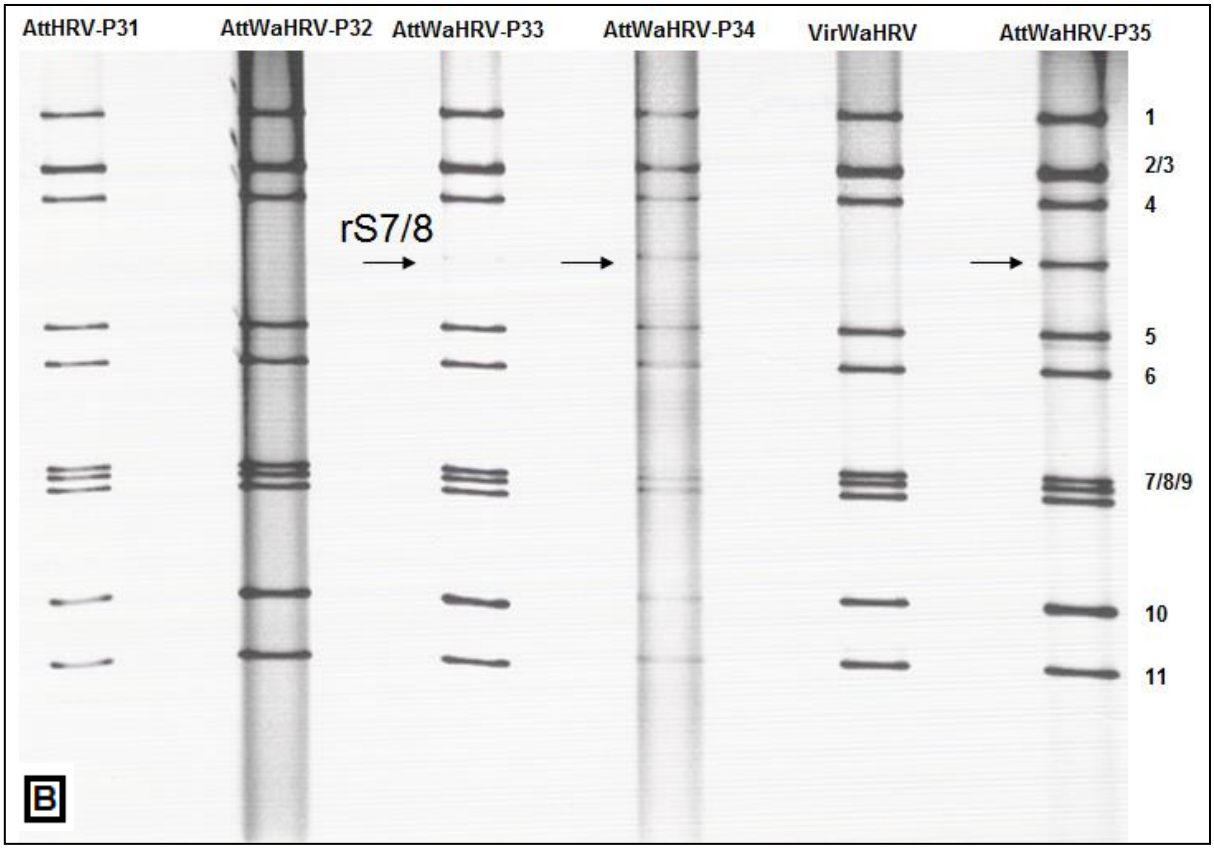
10

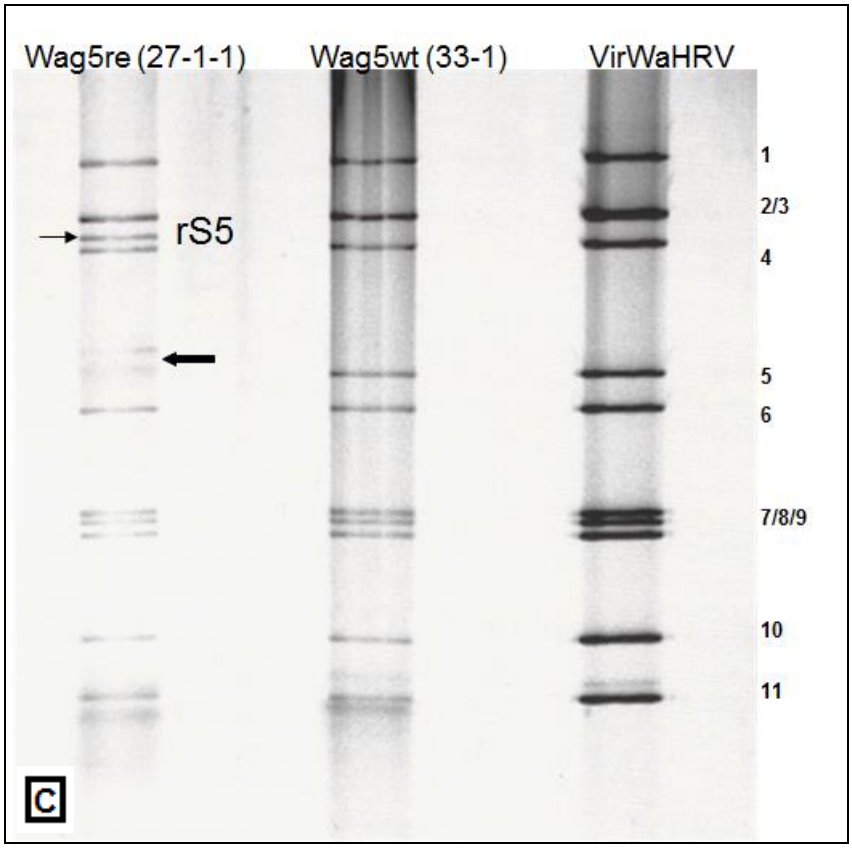
11

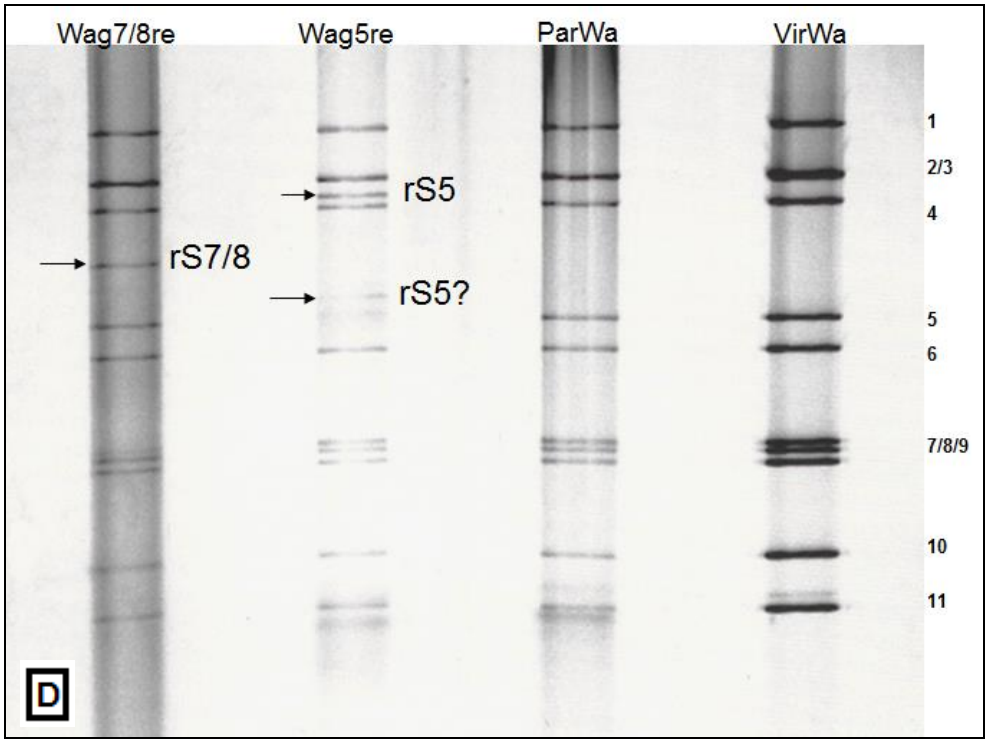

AttWaHRV-P24
AttWaHRV-P29
AttWaHRV-P34
(Wag7/8re)
VirWaHRV
rS7/8
1
2/3
4
5
6
7/8/9
10
11
A

12

13


AttHRV-P31
AttWaHRV-P32
AttWaHRV-P33
AttWaHRV-P34
VirWaHRV
AttWaHRV-P35
rS7/8
1
2/3
4
5
6
7/8/9
10
11
B





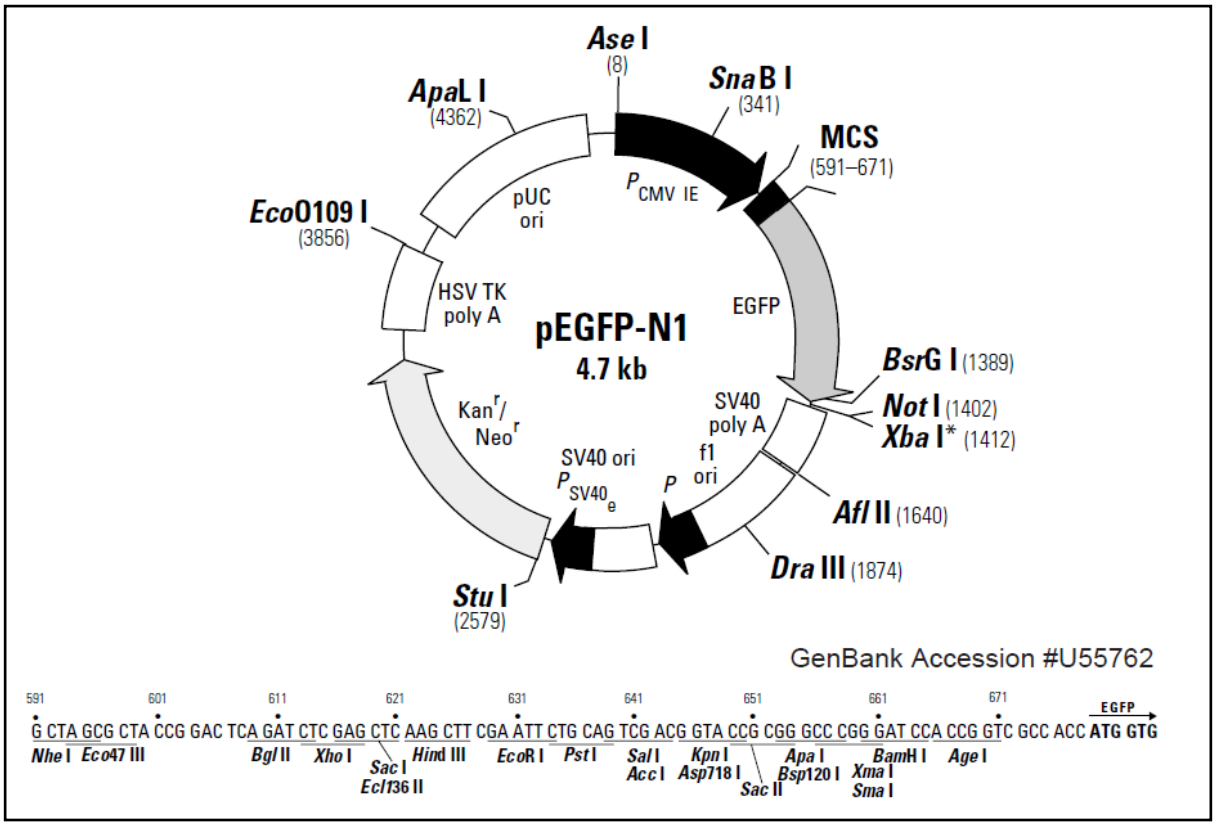
Article Supplementary Figure B1: Electropherotype patterns of the different rotavirus Wa variants**.** Electrophoresis was performed on 7.5% polyacrylamide gels (0.75mm thick) for 16 h at 20 mA at room temperature. RNA segments were visualized by silver staining. Arrows mark the rearranged genome segments (rS). **(A)** The electropherotype of the 29th passage

of AttWaHRV was still indistinguishable from the initial Wa HRV isolate. **(B)** Rearrangements in genome segments 7 and 8 were detected after the 33rd passage and became dominant at the 35th passage. **(C)** Electrophoretic pattern of Wag5re (27-1-1), Wag5wt (Wa33-1) and virulent, VirWa, human rotavirus strain. Small amount of original genome segment 5 is still present (pointed by thick black arrow), indicating that the aberrant gene 5 occurred gradually during passages in cell cultures. **(D)** Electropherotype patterns of all the different rotavirus Wa variants (Wag7/8re, Wag5re, ParWa and VirWa).

Article Supplementary Figure 2: ***Please see Supplementary Figure A.1 (Appendix A)***
Nucleotide sequence based phylogenetic trees of the genome segments encoding for the structural (VP1–VP4, VP6, VP7) and non-structural (NSP1–NSP5/6) proteins. The evolutionary history was inferred using the Neighbor-Joining method and the evolutionary distances were computed using the number of differences method and are in the units of the number of base differences per sequence. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. The arrows indicate the WaCS strain and the Wa, D, DS-1 and AU-1 reference strains are underlined. Scale bars are proportional to the phylogenetic distance.

APPENDIX C

pEGFP-N1 VECTOR



Supplementary Figure C.1: **The restriction map and multiple cloning site of the pEGFP-N1 plasmid (Clonetech).**

APPENDIX D

VECTOR SEQUENCES

Cloning vector pUC57 sequence

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GACATTAACCTATAAAAATAGGCGTATCACGAGGCCCTTTCGTC

Cloning vector pSMART LCAmp sequence

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phCMVDream sequence with BsmBI restriction sites

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180 GCCTCCGACCGGAGGCTTTTGACTTGATCGGCACGTAAGAGGTTCCAACTTTCACCATAATG

181 AAATAAGATCACTACCGGGCGTATTTTTTGAGTTATCGAGATTTTCAGGAGCTAAGGAAGCT

182 AAAATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCC

183 TGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCAC

184 GAGTGGGTTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTACGCCCCGAA

185 GAACGTTTTCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTAT

186 TGACGCCGGGCAAGAGCAACTCGGTCGCCGCATACACTATTCTCAGAATGACTTGGTTGAGT

187 ACTCACCAGTCACAGAAAAGCATCTCACGGATGGCATGACAGTAAGAGAATTATGCAGTGCT

188 GCCATAACCATGAGTGATAACACTGCGGCCAACTTACTTCTGGCAACGATCGGAGGACCGAA

189 GGAGCTAACCGCTTTTTTGCACAACATGGGGGATCATGTAACTCGCCTTGATCGTTGGGAAC

190 CGGAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACCACGATGCCTGTAGCAATGGCA

191 ACAACGTTGCGCAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAAT

192 AGACTGGATGGAGGCGGATAAAGTTGCAGGATCACTTCTGCGCTCGGCCCTCCCGGCTGGCT

193 GGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCACTG

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195 GGATGAACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGGTAATGAG

196 GGCCCAAATGTAATCACCTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTGCTGGCGTTTTTC

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201 TGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGT

202 CCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGA

203 GCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAG

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205 CTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGA

206 TTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATTTTCTACCGAAGAAAGGCCCAC

207 CCGTGAAGGTGAGCCAGTGAGTTGATTGCAGTCCAGTTACGCTGGAGTCTGAGGCTCGTCCT

208 GAATGATATCAAGCTT

APPENDIX E

DIFFERENT VISUALISATIONS OF IMMUNOSTAINED CELLS TRANSFECTED WITH ROTAVIRUS SA11 TRANSCRIPTS AND PLASMID DERIVED ROTAVIRUS SA11 GENOME SEGMENTS

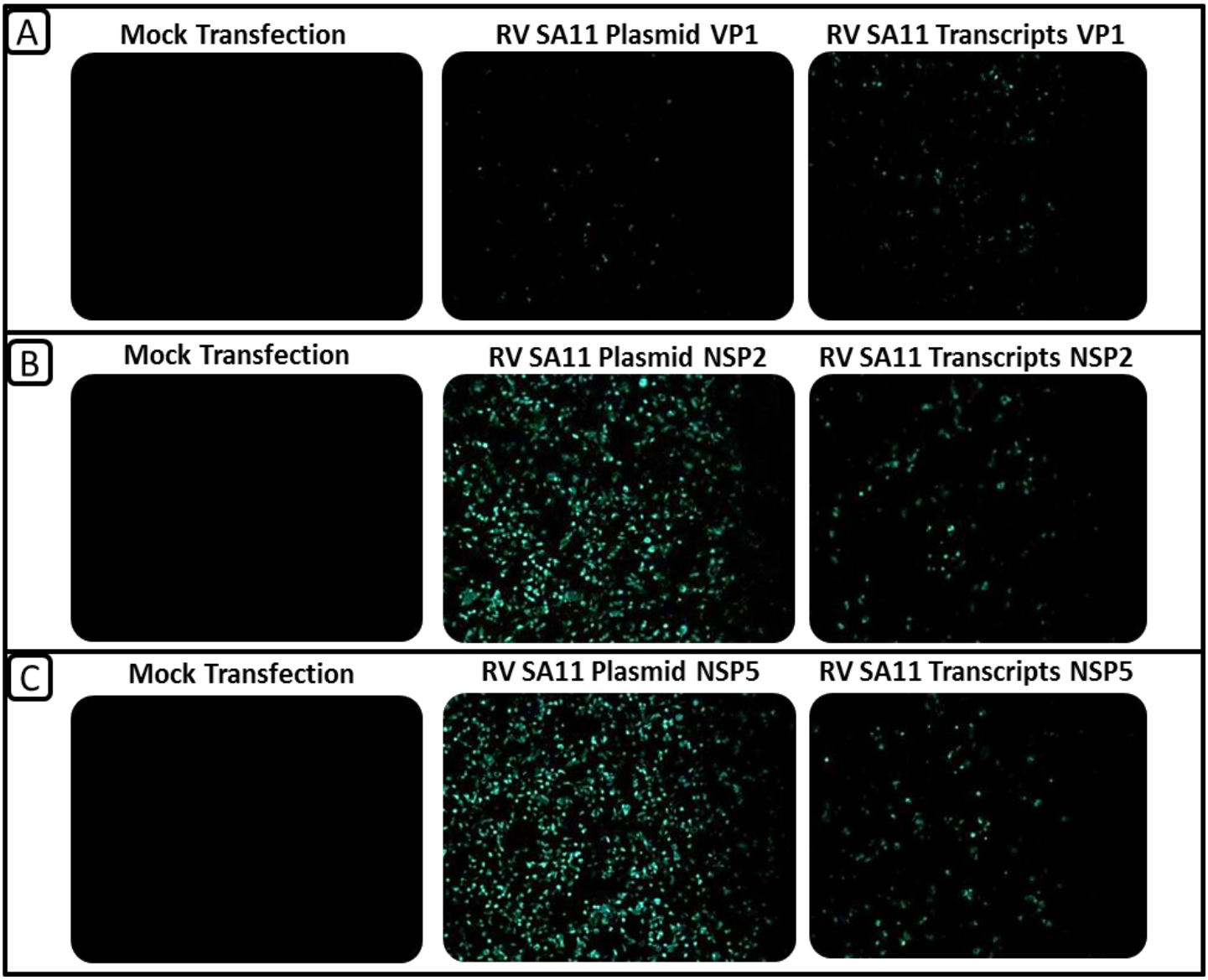

Figure E.1: ***Comparison of the iImmunological detection of expression between plasmid derived rotavirus protein expression and DLP derived rotavirus SA11 transcript expression in MA104 cells.*** *Cells were immunostained with antibodies showing the detection of rotavirus proteins, (A) VP1, (B) NSP2 and (C) NSP5 in MA104 cells following the transfection of the rotavirus SA11 plasmids or transcripts. The expression of rotavirus VP1, NSP2 and NSP5 were determined by immunofluorescence microscopy.* ***No visual enhancements on photos.***

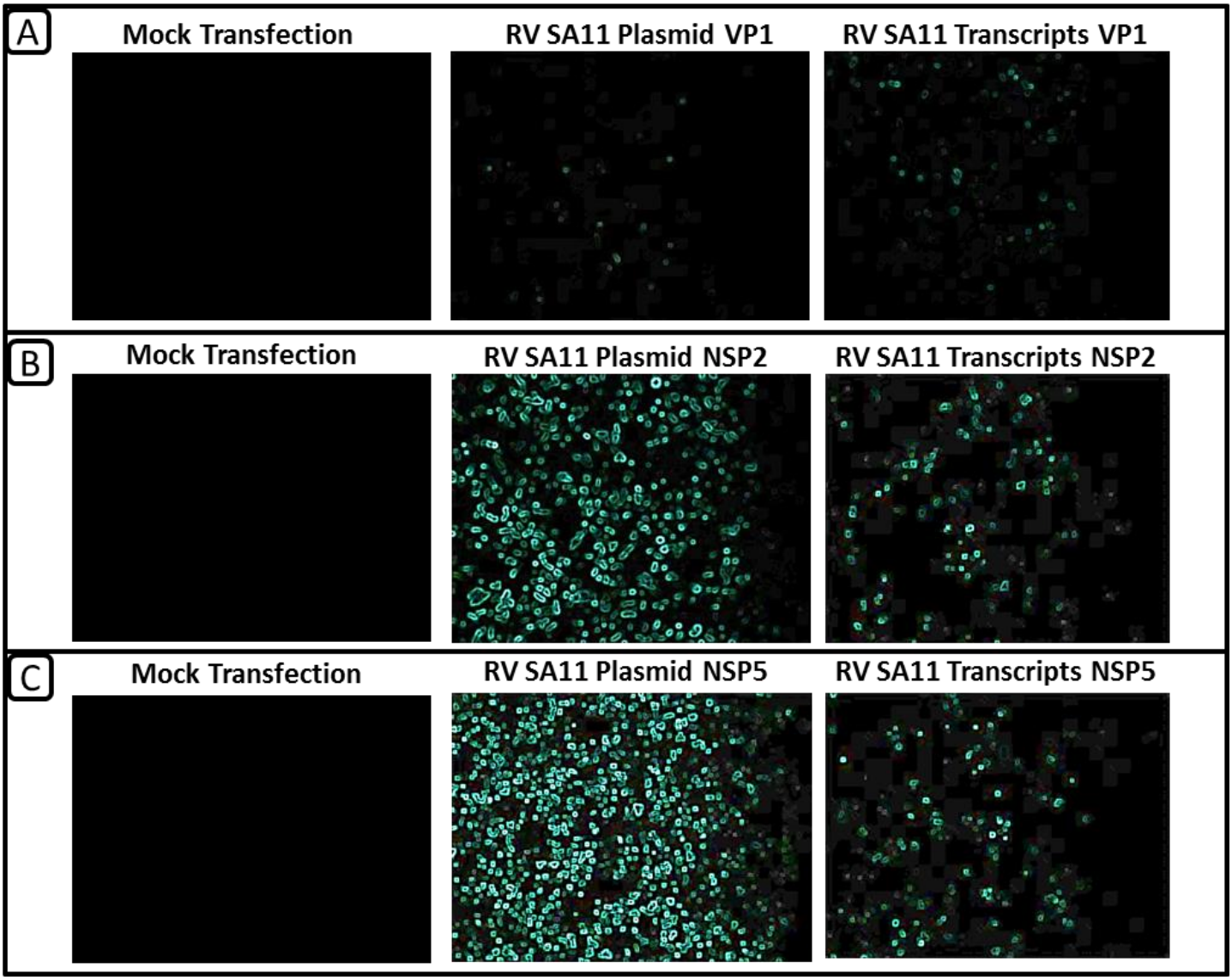

Figure E.2: ***Comparison of the iImmunological detection of expression between plasmid derived rotavirus protein expression and DLP derived rotavirus SA11 transcript expression in MA104 cells.*** *Cells were immunostained with antibodies showing the detection of rotavirus proteins, (A) VP1, (B) NSP2 and (C) NSP5 in MA104 cells following the transfection of the rotavirus SA11 plasmids or transcripts. The expression of rotavirus VP1, NSP2 and NSP5 were determined by immunofluorescence microscopy.* ***Visually enhancement: Photo sharpened 100%; glow filter applied (highlights pixels that is in stark contrast to the perceived background); saturation 400%; contrast -40%; brightness 40%. MS PowerPoint Professional Plus 2010 used for visual enhancements.***

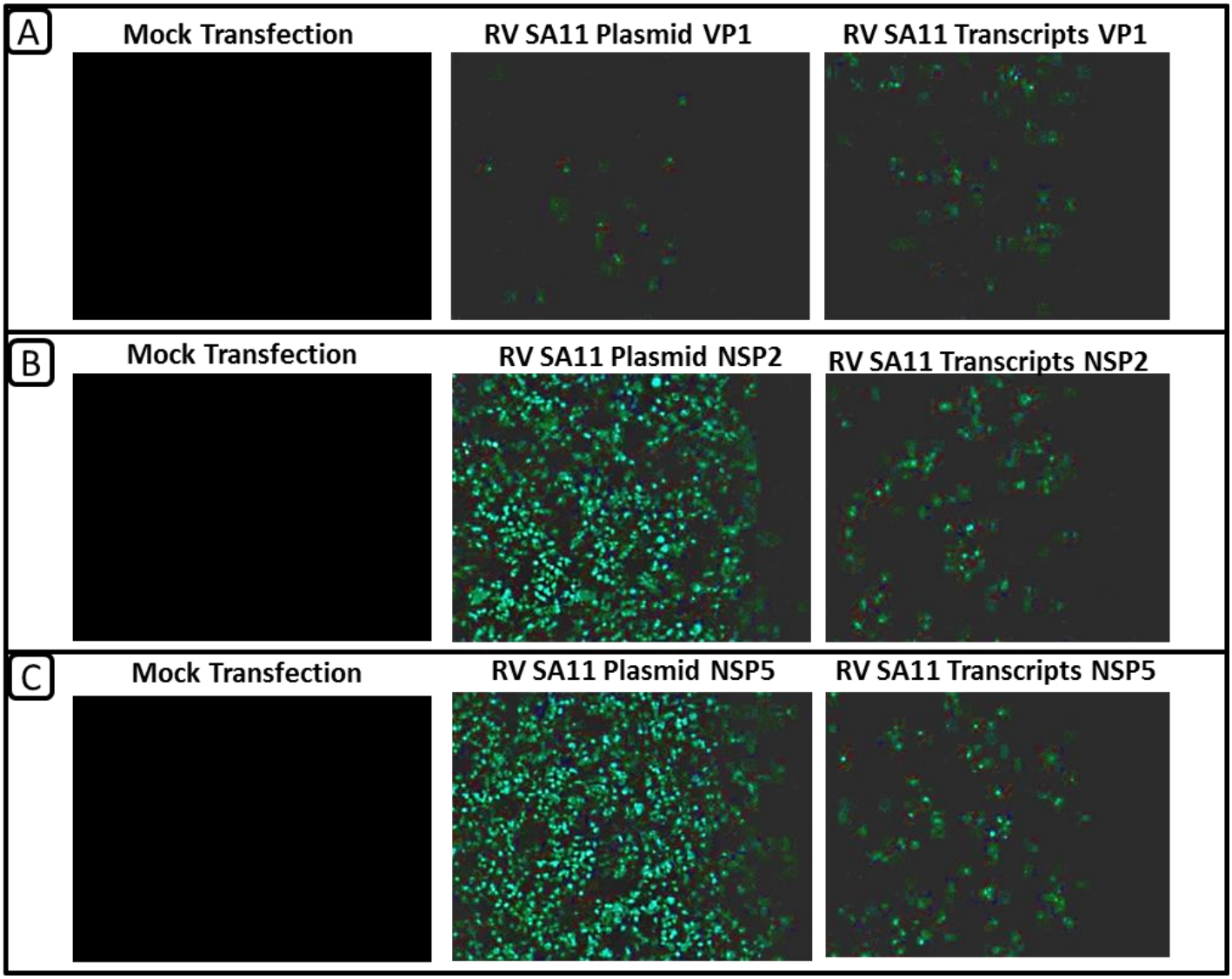

Figure E.3: ***Comparison of the iImmunological detection of expression between plasmid derived rotavirus protein expression and DLP derived rotavirus SA11 transcript expression in MA104 cells.*** *Cells were immunostained with antibodies showing the detection of rotavirus proteins, (A) VP1, (B) NSP2 and (C) NSP5 in MA104 cells following the transfection of the rotavirus SA11 plasmids or transcripts. The expression of rotavirus VP1, NSP2 and NSP5 were determined by immunofluorescence microscopy.* ***Visually enhancement: Photo sharpened 100%; photocopy filter applied; saturation 400%; contrast 40%; brightness 40%; transparency 0%; smoothness 0%. MS PowerPoint Professional Plus 2010 used for visual enhancements.***

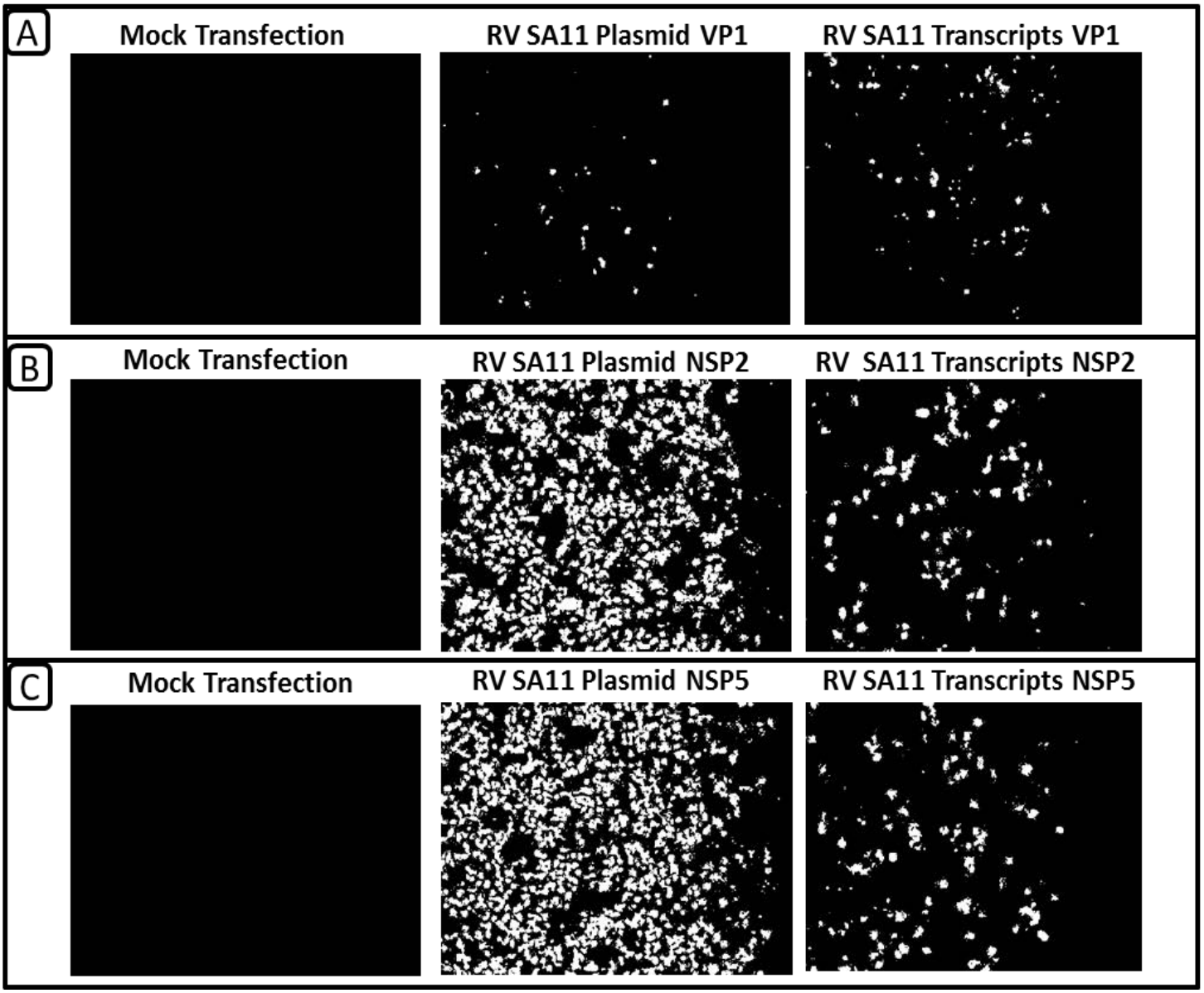

Figure E.4: Comparison of the iImmunological detection of expression between plasmid derived rotavirus protein expression and DLP derived rotavirus SA11 transcript expression in MA104 cells. *Cells were immunostained with antibodies showing the detection of rotavirus proteins, (A) VP1, (B) NSP2 and (C) NSP5 in MA104 cells following the transfection of the rotavirus SA11 plasmids or transcripts. The expression of rotavirus VP1, NSP2 and NSP5 were determined by immunofluorescence microscopy.* ***Visually enhancement: Photo sharpened 100%; photocopy filter applied; saturation 400%; contrast 20%; brightness 40%; transparency 93%; detail 100%; Temperature 1200 K; black and white high contrast filter. MS PowerPoint Professional Plus 2010 used for visual enhancements.***

Acknowledgement: I express gratitude to A. Wentzel for enhancing the photos of cells that were immunostained with antibodies to better show the expression of different rotavirus proteins.

APPENDIX F

STATISTICAL ANALYSIS, MULTIPLE COMPARISON TEST RESULTS, ADJUSTED P VALUES AND ONE-WAY ANOVAS

Supplementary Table F1: RM one-way ANOVA of IFN Alpha: ANOVA

	Data Set-A	Data Set-B	Data Set-C	Data Set-D	Data Set-E
Table Analyzed	IFN Alpha				
Repeated measures ANOVA summary					
Assume sphericity?	No				
F	155.7				
P value	0.0004				
P value summary	***				
Statistically significant (P < 0.05)?	Yes				
Geisser-Greenhouse's epsilon	0.08791				
R square	0.9873				
Was the matching effective?					
F	1.747				
P value	0.1873				
P value summary	ns				
Is there significant matching (P < 0.05)?	No				
R square	0.001107				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	1471	20	73.56	F (1.758, 3.516) = 155.7	P = 0.0004
Individual (between rows)	1.651	2	0.8254	F (2, 40) = 1.747	P = 0.1873
Residual (random)	18.9	40	0.4724		
Total	1492	62			
Data summary					
Number of treatments (columns)	21				
Number of subjects (rows)	3				

Supplementary Table F2: RM one-way ANOVA of IFN Alpha: Multiple comparison

	Data Set-A	Data Set-B	Data Set-C	Data Set-D	Data Set-E
Number of families	1				

Number of comparisons per family	20				
Alpha	0.05				
Dunnett's multiple comparisons test	**Mean Diff.**	**95% CI of diff.**	**Significant?**	**Summary**	**Adjusted P Value**
Mock Transfection vs. Cell Control	-0.7	-6.341 to 4.941	No	ns	0.9083
Mock Transfection vs. SA11 infection	-13.33	-21.41 to -5.255	Yes	*	0.0188
Mock Transfection vs. SA11 mRNA transcripts	-8.233	-14.65 to -1.819	Yes	*	0.0308
Mock Transfection vs. Wa GS1 Plasmid (VP1)	3.3	-0.2096 to 6.810	No	ns	0.0563
Mock Transfection vs. Wa GS2 Plasmid (VP2)	7.333	5.554 to 9.112	Yes	**	0.003
Mock Transfection vs. Wa GS3 Plasmid (VP3)	-4	-8.146 to 0.1464	No	ns	0.0536
Mock Transfection vs. Wa GS4 Plasmid (VP4)	0.8	-3.221 to 4.821	No	ns	0.6679
Mock Transfection vs. Wa GS5 Plasmid (NSP1)	0.4	-0.1066 to 0.9066	No	ns	0.0786
Mock Transfection vs. Wa GS6 Plasmid (VP6)	-1.5	-4.181 to 1.181	No	ns	0.1486
Mock Transfection vs. Wa GS7 Plasmid (NSP3)	-1.867	-3.976 to 0.2423	No	ns	0.0632
Mock Transfection vs. Wa GS8 Plasmid (NSP2)	-1.667	-6.481 to 3.148	No	ns	0.3327
Mock Transfection vs. Wa GS9 Plasmid (VP7)	-1.067	-4.284 to 2.150	No	ns	0.355
Mock Transfection vs. Wa GS10 Plasmid (NSP4)	-2.233	-3.781 to -0.6857	Yes	*	0.0244
Mock Transfection vs. Wa GS11 Plasmid (NSP5/6)	-4.667	-7.216 to -2.117	Yes	*	0.0153
Mock Transfection vs. All Wa Plasmids (GS1-11)	0.8667	0.09287 to 1.640	Yes	*	0.0401
Mock Transfection vs. SA11 Alpha Plasmid (VP1 + NSP2 + NSP5/6)	-5.167	-7.957 to -2.377	Yes	*	0.0149
Mock Transfection vs. SA11 Beta Plasmid (VP2 + VP3)	7.7	3.744 to 11.66	Yes	*	0.0135
Mock Transfection vs. SA11 Gamma Plasmid (VP4 + NSP1 + VP6)	5.167	3.997 to 6.337	Yes	**	0.0026
Mock Transfection vs. SA11 Delta Plasmid (NSP4 + VP7 + NSP3)	-3.2	-7.156 to 0.7564	No	ns	0.0751
Mock Transfection vs. All SA11 Plasmids (Alpha-Delta)	4.233	3.941 to 4.526	Yes	***	0.0003
Test details	**Mean 1**	**Mean 2**	**Mean Diff.**	**SE of diff.**	**n1**
Mock Transfection vs. Cell Control	0.06667	0.7667	-0.7	0.6429	3
Mock Transfection vs. SA11 infection	0.06667	13.4	-13.33	0.9207	3
Mock Transfection vs. SA11 mRNA transcripts	0.06667	8.3	-8.233	0.7311	3
Mock Transfection vs. Wa GS1 Plasmid (VP1)	0.06667	-3.233	3.3	0.4	3
Mock Transfection vs. Wa GS2 Plasmid (VP2)	0.06667	-7.267	7.333	0.2028	3
Mock Transfection vs. Wa GS3 Plasmid (VP3)	0.06667	4.067	-4	0.4726	3

Mock Transfection vs. Wa GS4 Plasmid (VP4)	0.06667	-0.7333	0.8	0.4583	3
Mock Transfection vs. Wa GS5 Plasmid (NSP1)	0.06667	-0.3333	0.4	0.05774	3
Mock Transfection vs. Wa GS6 Plasmid (VP6)	0.06667	1.567	-1.5	0.3055	3
Mock Transfection vs. Wa GS7 Plasmid (NSP3)	0.06667	1.933	-1.867	0.2404	3
Mock Transfection vs. Wa GS8 Plasmid (NSP2)	0.06667	1.733	-1.667	0.5487	3
Mock Transfection vs. Wa GS9 Plasmid (VP7)	0.06667	1.133	-1.067	0.3667	3
Mock Transfection vs. Wa GS10 Plasmid (NSP4)	0.06667	2.3	-2.233	0.1764	3
Mock Transfection vs. Wa GS11 Plasmid (NSP5/6)	0.06667	4.733	-4.667	0.2906	3
Mock Transfection vs. All Wa Plasmids (GS1-11)	0.06667	-0.8	0.8667	0.08819	3
Mock Transfection vs. SA11 Alpha Plasmid (VP1 + NSP2 + NSP5/6)	0.06667	5.233	-5.167	0.318	3
Mock Transfection vs. SA11 Beta Plasmid (VP2 + VP3)	0.06667	-7.633	7.7	0.4509	3
Mock Transfection vs. SA11 Gamma Plasmid (VP4 + NSP1 + VP6)	0.06667	-5.1	5.167	0.1333	3
Mock Transfection vs. SA11 Delta Plasmid (NSP4 + VP7 + NSP3)	0.06667	3.267	-3.2	0.4509	3
Mock Transfection vs. All SA11 Plasmids (Alpha-Delta)	0.06667	-4.167	4.233	0.03333	3

Supplementary Table F3: RM one-way ANOVA of IFN Beta: ANOVA

	Data Set-A	Data Set-B	Data Set-C	Data Set-D	Data Set-E
Table Analyzed	IFN Beta				
Repeated measures ANOVA summary					
Assume sphericity?	No				
F	242.1				
P value	0.0002				
P value summary	***				
Statistically significant (P < 0.05)?	Yes				
Geisser-Greenhouse's epsilon	0.08396				
R square	0.9918				
Was the matching effective?					
F	0.1985				
P value	0.8208				
P value summary	ns				
Is there significant matching (P < 0.05)?	No				
R square	8.13E-05				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	2525	20	126.3	F (1.679, 3.359) =	P = 0.0002

				242.1	
Individual (between rows)	0.207	2	0.1035	F (2, 40) = 0.1985	P = 0.8208
Residual (random)	20.86	40	0.5215		
Total	2546	62			
Data summary					
Number of treatments (columns)	21				
Number of subjects (rows)	3				

Supplementary Table F4: RM one-way ANOVA of IFN Beta: Multiple comparisons

	Data Set-A	Data Set-B	Data Set-C	Data Set-D	Data Set-E
Number of families	1				
Number of comparisons per family	20				
Alpha	0.05				
Dunnett's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
Mock Transfection vs. Cell Control	4.97E-09	-2.632 to 2.632	No	ns	> 0.9999
Mock Transfection vs. SA11 infection	-12.53	-14.08 to -10.99	Yes	***	0.0008
Mock Transfection vs. SA11 mRNA transcripts	-20.2	-32.39 to -8.011	Yes	*	0.0186
Mock Transfection vs. Wa GS1 Plasmid (VP1)	4.533	-0.5911 to 9.658	No	ns	0.0633
Mock Transfection vs. Wa GS2 Plasmid (VP2)	7.533	3.166 to 11.90	Yes	*	0.0172
Mock Transfection vs. Wa GS3 Plasmid (VP3)	-5.133	-10.21 to -0.05922	Yes	*	0.0489
Mock Transfection vs. Wa GS4 Plasmid (VP4)	1.333	-2.401 to 5.067	No	ns	0.3172
Mock Transfection vs. Wa GS5 Plasmid (NSP1)	0.7333	-1.551 to 3.018	No	ns	0.3723
Mock Transfection vs. Wa GS6 Plasmid (VP6)	-2.167	-4.561 to 0.2273	No	ns	0.0606
Mock Transfection vs. Wa GS7 Plasmid (NSP3)	-2.733	-4.362 to -1.105	Yes	*	0.0181
Mock Transfection vs. Wa GS8 Plasmid (NSP2)	-2.467	-6.592 to 1.659	No	ns	0.1318
Mock Transfection vs. Wa GS9 Plasmid (VP7)	-1.4	-4.910 to 2.110	No	ns	0.2659
Mock Transfection vs. Wa GS10 Plasmid (NSP4)	-2.6	-7.432 to 2.232	No	ns	0.1594
Mock Transfection vs. Wa GS11 Plasmid (NSP5/6)	-6.1	-8.920 to -3.280	Yes	*	0.011
Mock Transfection vs. All Wa Plasmids (GS1-11)	0.6	-8.012 to 9.212	No	ns	0.9959
Mock Transfection vs. SA11 Alpha Plasmid	-6.8	-10.82 to -	Yes	*	0.0179

(VP1 + NSP2 + NSP5/6)		2.779			
Mock Transfection vs. SA11 Beta Plasmid (VP2 + VP3)	7.467	2.926 to 12.01	Yes	*	0.0189
Mock Transfection vs. SA11 Gamma Plasmid (VP4 + NSP1 + VP6)	4.7	-1.767 to 11.17	No	ns	0.0919
Mock Transfection vs. SA11 Delta Plasmid (NSP4 + VP7 + NSP3)	-1.733	-4.990 to 1.523	No	ns	0.1625
Mock Transfection vs. All SA11 Plasmids (Alpha-Delta)	5.2	2.036 to 8.364	Yes	*	0.0189

Test details	**Mean 1**	**Mean 2**	**Mean Diff.**	**SE of diff.**	**n1**
Mock Transfection vs. Cell Control	0.1667	0.1667	4.97E-09	0.3	3
Mock Transfection vs. SA11 infection	0.1667	12.7	-12.53	0.1764	3
Mock Transfection vs. SA11 mRNA transcripts	0.1667	20.37	-20.2	1.389	3
Mock Transfection vs. Wa GS1 Plasmid (VP1)	0.1667	-4.367	4.533	0.584	3
Mock Transfection vs. Wa GS2 Plasmid (VP2)	0.1667	-7.367	7.533	0.4978	3
Mock Transfection vs. Wa GS3 Plasmid (VP3)	0.1667	5.3	-5.133	0.5783	3
Mock Transfection vs. Wa GS4 Plasmid (VP4)	0.1667	-1.167	1.333	0.4256	3
Mock Transfection vs. Wa GS5 Plasmid (NSP1)	0.1667	-0.5667	0.7333	0.2603	3
Mock Transfection vs. Wa GS6 Plasmid (VP6)	0.1667	2.333	-2.167	0.2728	3
Mock Transfection vs. Wa GS7 Plasmid (NSP3)	0.1667	2.9	-2.733	0.1856	3
Mock Transfection vs. Wa GS8 Plasmid (NSP2)	0.1667	2.633	-2.467	0.4702	3
Mock Transfection vs. Wa GS9 Plasmid (VP7)	0.1667	1.567	-1.4	0.4	3
Mock Transfection vs. Wa GS10 Plasmid (NSP4)	0.1667	2.767	-2.6	0.5508	3
Mock Transfection vs. Wa GS11 Plasmid (NSP5/6)	0.1667	6.267	-6.1	0.3215	3
Mock Transfection vs. All Wa Plasmids (GS1-11)	0.1667	-0.4333	0.6	0.9815	3
Mock Transfection vs. SA11 Alpha Plasmid (VP1 + NSP2 + NSP5/6)	0.1667	6.967	-6.8	0.4583	3
Mock Transfection vs. SA11 Beta Plasmid (VP2 + VP3)	0.1667	-7.3	7.467	0.5175	3
Mock Transfection vs. SA11 Gamma Plasmid (VP4 + NSP1 + VP6)	0.1667	-4.533	4.7	0.7371	3
Mock Transfection vs. SA11 Delta Plasmid (NSP4 + VP7 + NSP3)	0.1667	1.9	-1.733	0.3712	3
Mock Transfection vs. All SA11 Plasmids (Alpha-Delta)	0.1667	-5.033	5.2	0.3606	3

Supplementary Table F5: RM one-way ANOVA of IFN Lambda: ANOVA

	Data Set-A	**Data Set-B**	**Data Set-C**	**Data Set-D**	**Data Set-E**
Table Analyzed	IFN Lambda				
Repeated measures ANOVA summary					

Assume sphericity?	No				
F	232.4				
P value	0.0006				
P value summary	***				
Statistically significant (P < 0.05)?	Yes				
Geisser-Greenhouse's epsilon	0.07472				
R square	0.9915				
Was the matching effective?					
F	3.739				
P value	0.0325				
P value summary	*				
Is there significant matching (P < 0.05)?	Yes				
R square	0.001593				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	1476	20	73.78	F (1.494, 2.989) = 232.4	P = 0.0006
Individual (between rows)	2.374	2	1.187	F (2, 40) = 3.739	P = 0.0325
Residual (random)	12.7	40	0.3175		
Total	1491	62			
Data summary					
Number of treatments (columns)	21				
Number of subjects (rows)	3				

Supplementary Table F6: RM one-way ANOVA of IFN Lambda: Multiple comparisons

	Data Set-A	Data Set-B	Data Set-C	Data Set-D	Data Set-E
Number of families	1				
Number of comparisons per family	20				
Alpha	0.05				
Dunnett's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
Mock Transfection vs. Cell Control	-0.1	-2.781 to 2.581	No	ns	0.9997
Mock Transfection vs. SA11 infection	-4.367	-8.101 to -0.6327	Yes	*	0.0369
Mock Transfection vs. SA11 mRNA transcripts	-18.63	-25.05 to -12.22	Yes	**	0.0061

Mock Transfection vs. Wa GS1 Plasmid (VP1)	-3.167	-4.221 to -2.112	Yes	**	0.0057
Mock Transfection vs. Wa GS2 Plasmid (VP2)	4.7	0.05723 to 9.343	Yes	*	0.0488
Mock Transfection vs. Wa GS3 Plasmid (VP3)	3.8	2.787 to 4.813	Yes	**	0.0037
Mock Transfection vs. Wa GS4 Plasmid (VP4)	1.933	-3.141 to 7.007	No	ns	0.2864
Mock Transfection vs. Wa GS5 Plasmid (NSP1)	0.8667	-1.923 to 3.657	No	ns	0.3905
Mock Transfection vs. Wa GS6 Plasmid (VP6)	-2.633	-3.803 to -1.463	Yes	*	0.0101
Mock Transfection vs. Wa GS7 Plasmid (NSP3)	-1.767	-4.820 to 1.287	No	ns	0.1399
Mock Transfection vs. Wa GS8 Plasmid (NSP2)	-2.633	-4.096 to -1.171	Yes	*	0.0158
Mock Transfection vs. Wa GS9 Plasmid (VP7)	-2.967	-4.885 to -1.049	Yes	*	0.0213
Mock Transfection vs. Wa GS10 Plasmid (NSP4)	-2.633	-5.514 to 0.2471	No	ns	0.0594
Mock Transfection vs. Wa GS11 Plasmid (NSP5/6)	-6.233	-10.30 to -2.170	Yes	*	0.0217
Mock Transfection vs. All Wa Plasmids (GS1-11)	-0.1	-4.056 to 3.856	No	ns	0.9998
Mock Transfection vs. SA11 Alpha Plasmid (VP1 + NSP2 + NSP5/6)	-6.4	-9.481 to -3.319	Yes	*	0.0119
Mock Transfection vs. SA11 Beta Plasmid (VP2 + VP3)	-1.033	-2.308 to 0.2415	No	ns	0.0748
Mock Transfection vs. SA11 Gamma Plasmid (VP4 + NSP1 + VP6)	0.8	-3.221 to 4.821	No	ns	0.6679
Mock Transfection vs. SA11 Delta Plasmid (NSP4 + VP7 + NSP3)	-2.667	-5.457 to 0.1233	No	ns	0.0546
Mock Transfection vs. All SA11 Plasmids (Alpha-Delta)	4.833	1.460 to 8.206	Yes	*	0.0248

Test details	**Mean 1**	**Mean 2**	**Mean Diff.**	**SE of diff.**	**n1**
Mock Transfection vs. Cell Control	-0.1667	-0.06667	-0.1	0.3055	3
Mock Transfection vs. SA11 infection	-0.1667	4.2	-4.367	0.4256	3
Mock Transfection vs. SA11 mRNA transcripts	-0.1667	18.47	-18.63	0.7311	3
Mock Transfection vs. Wa GS1 Plasmid (VP1)	-0.1667	3	-3.167	0.1202	3
Mock Transfection vs. Wa GS2 Plasmid (VP2)	-0.1667	-4.867	4.7	0.5292	3
Mock Transfection vs. Wa GS3 Plasmid (VP3)	-0.1667	-3.967	3.8	0.1155	3
Mock Transfection vs. Wa GS4 Plasmid (VP4)	-0.1667	-2.1	1.933	0.5783	3
Mock Transfection vs. Wa GS5 Plasmid (NSP1)	-0.1667	-1.033	0.8667	0.318	3
Mock Transfection vs. Wa GS6 Plasmid (VP6)	-0.1667	2.467	-2.633	0.1333	3
Mock Transfection vs. Wa GS7 Plasmid (NSP3)	-0.1667	1.6	-1.767	0.348	3
Mock Transfection vs. Wa GS8 Plasmid (NSP2)	-0.1667	2.467	-2.633	0.1667	3
Mock Transfection vs. Wa GS9 Plasmid (VP7)	-0.1667	2.8	-2.967	0.2186	3
Mock Transfection vs. Wa GS10 Plasmid (NSP4)	-0.1667	2.467	-2.633	0.3283	3
Mock Transfection vs. Wa GS11 Plasmid (NSP5/6)	-0.1667	6.067	-6.233	0.4631	3
Mock Transfection vs. All Wa Plasmids (GS1-11)	-0.1667	-0.06667	-0.1	0.4509	3

Mock Transfection vs. SA11 Alpha Plasmid (VP1 + NSP2 + NSP5/6)	-0.1667	6.233	-6.4	0.3512	3
Mock Transfection vs. SA11 Beta Plasmid (VP2 + VP3)	-0.1667	0.8667	-1.033	0.1453	3
Mock Transfection vs. SA11 Gamma Plasmid (VP4 + NSP1 + VP6)	-0.1667	-0.9667	0.8	0.4583	3
Mock Transfection vs. SA11 Delta Plasmid (NSP4 + VP7 + NSP3)	-0.1667	2.5	-2.667	0.318	3
Mock Transfection vs. All SA11 Plasmids (Alpha-Delta)	-0.1667	-5	4.833	0.3844	3

Supplementary Table F7: RM one-way ANOVA of CXCL10:ANOVA

	Data Set-A	Data Set-B	Data Set-C	Data Set-D	Data Set-E
Table Analyzed	CXCL10				
Repeated measures ANOVA summary					
Assume sphericity?	No				
F	220.2				
P value	0.0003				
P value summary	***				
Statistically significant (P < 0.05)?	Yes				
Geisser-Greenhouse's epsilon	0.08369				
R square	0.991				
Was the matching effective?					
F	0.5596				
P value	0.5758				
P value summary	ns				
Is there significant matching (P < 0.05)?	No				
R square	0.0002517				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	1845	20	92.26	F (1.674, 3.348) = 220.2	P = 0.0003
Individual (between rows)	0.4689	2	0.2344	F (2, 40) = 0.5596	P = 0.5758
Residual (random)	16.76	40	0.4189		
Total	1863	62			
Data summary					
Number of treatments (columns)	21				
Number of subjects (rows)	3				

Supplementary Table F8: RM one-way ANOVA of CXCL10: Multiple comparisons

	Data Set-A	Data Set-B	Data Set-C	Data Set-D	Data Set-E
Number of families	1				
Number of comparisons per family	20				
Alpha	0.05				
Dunnett's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	
Mock Transfection vs. Cell Control	0.5667	-6.236 to 7.369	No	ns	
Mock Transfection vs. SA11 infection	-8.367	-19.65 to 2.919	No	ns	
Mock Transfection vs. SA11 mRNA transcripts	-16.17	-17.95 to -14.39	Yes	***	
Mock Transfection vs. Wa GS1 Plasmid (VP1)	3.033	-2.728 to 8.794	No	ns	
Mock Transfection vs. Wa GS2 Plasmid (VP2)	7.7	3.554 to 11.85	Yes	*	
Mock Transfection vs. Wa GS3 Plasmid (VP3)	-1.5	-7.579 to 4.579	No	ns	
Mock Transfection vs. Wa GS4 Plasmid (VP4)	2.133	-3.447 to 7.713	No	ns	
Mock Transfection vs. Wa GS5 Plasmid (NSP1)	1.833	-4.038 to 7.705	No	ns	
Mock Transfection vs. Wa GS6 Plasmid (VP6)	-0.9	-2.240 to 0.4403	No	ns	
Mock Transfection vs. Wa GS7 Plasmid (NSP3)	-1.733	-8.067 to 4.600	No	ns	
Mock Transfection vs. Wa GS8 Plasmid (NSP2)	-0.8667	-5.085 to 3.351	No	ns	
Mock Transfection vs. Wa GS9 Plasmid (VP7)	2.6	-0.2204 to 5.420	No	ns	
Mock Transfection vs. Wa GS10 Plasmid (NSP4)	-0.8	-7.837 to 6.237	No	ns	
Mock Transfection vs. Wa GS11 Plasmid (NSP5/6)	-4.467	-10.66 to 1.724	No	ns	
Mock Transfection vs. All Wa Plasmids (GS1-11)	1.267	-1.787 to 4.320	No	ns	
Mock Transfection vs. SA11 Alpha Plasmid (VP1 + NSP2 + NSP5/6)	-3.933	-8.028 to 0.1612	No	ns	
Mock Transfection vs. SA11 Beta Plasmid (VP2 + VP3)	5.533	0.1168 to 10.95	Yes	*	
Mock Transfection vs. SA11 Gamma Plasmid (VP4 + NSP1 + VP6)	8.633	4.266 to 13.00	Yes	*	
Mock Transfection vs. SA11 Delta Plasmid (NSP4 + VP7 + NSP3)	5.133	-2.720 to 12.99	No	ns	
Mock Transfection vs. All SA11 Plasmids (Alpha-Delta)	5.033	-0.8379 to 10.90	No	ns	
Test details	**Mean 1**	**Mean 2**	**Mean Diff.**	**SE of diff.**	**n1**

Mock Transfection vs. Cell Control	0.7333	0.1667	0.5667	0.7753	3
Mock Transfection vs. SA11 infection	0.7333	9.1	-8.367	1.286	3
Mock Transfection vs. SA11 mRNA transcripts	0.7333	16.9	-16.17	0.2028	3
Mock Transfection vs. Wa GS1 Plasmid (VP1)	0.7333	-2.3	3.033	0.6566	3
Mock Transfection vs. Wa GS2 Plasmid (VP2)	0.7333	-6.967	7.7	0.4726	3
Mock Transfection vs. Wa GS3 Plasmid (VP3)	0.7333	2.233	-1.5	0.6928	3
Mock Transfection vs. Wa GS4 Plasmid (VP4)	0.7333	-1.4	2.133	0.636	3
Mock Transfection vs. Wa GS5 Plasmid (NSP1)	0.7333	-1.1	1.833	0.6692	3
Mock Transfection vs. Wa GS6 Plasmid (VP6)	0.7333	1.633	-0.9	0.1528	3
Mock Transfection vs. Wa GS7 Plasmid (NSP3)	0.7333	2.467	-1.733	0.7219	3
Mock Transfection vs. Wa GS8 Plasmid (NSP2)	0.7333	1.6	-0.8667	0.4807	3
Mock Transfection vs. Wa GS9 Plasmid (VP7)	0.7333	-1.867	2.6	0.3215	3
Mock Transfection vs. Wa GS10 Plasmid (NSP4)	0.7333	1.533	-0.8	0.8021	3
Mock Transfection vs. Wa GS11 Plasmid (NSP5/6)	0.7333	5.2	-4.467	0.7055	3
Mock Transfection vs. All Wa Plasmids (GS1-11)	0.7333	-0.5333	1.267	0.348	3
Mock Transfection vs. SA11 Alpha Plasmid (VP1 + NSP2 + NSP5/6)	0.7333	4.667	-3.933	0.4667	3
Mock Transfection vs. SA11 Beta Plasmid (VP2 + VP3)	0.7333	-4.8	5.533	0.6173	3
Mock Transfection vs. SA11 Gamma Plasmid (VP4 + NSP1 + VP6)	0.7333	-7.9	8.633	0.4978	3
Mock Transfection vs. SA11 Delta Plasmid (NSP4 + VP7 + NSP3)	0.7333	-4.4	5.133	0.895	3
Mock Transfection vs. All SA11 Plasmids (Alpha-Delta)	0.7333	-4.3	5.033	0.6692	3

Supplementary Table F9: RM one-way ANOVA of TNF: ANOVA

	Data Set-A	Data Set-B	Data Set-C	Data Set-D	Data Set-E
Table Analyzed	TNF				
Repeated measures ANOVA summary					
Assume sphericity?	No				
F	110.5				
P value	0.0003				
P value summary	***				
Statistically significant (P < 0.05)?	Yes				
Geisser-Greenhouse's epsilon	0.09986				
R square	0.9822				
Was the matching effective?					
F	1.291				

P value	0.2861				
P value summary	ns				
Is there significant matching (P < 0.05)?	No				
R square	0.001147				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	557.2	20	27.86	F (1.997, 3.995) = 110.5	P = 0.0003
Individual (between rows)	0.6514	2	0.3257	F (2, 40) = 1.291	P = 0.2861
Residual (random)	10.09	40	0.2522		
Total	567.9	62			
Data summary					
Number of treatments (columns)	21				
Number of subjects (rows)	3				

Supplementary Table F10: RM one-way ANOVA of TNF: Multiple comparisons

	Data Set-A	Data Set-B	Data Set-C	Data Set-D	Data Set-E
Number of families	1				
Number of comparisons per family	20				
Alpha	0.05				
Dunnett's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	
Mock Transfection vs. Cell Control	0.1	-1.655 to 1.855	No	ns	
Mock Transfection vs. SA11 infection	-2.3	-2.807 to -1.793	Yes	**	
Mock Transfection vs. SA11 mRNA transcripts	-2.4	-3.740 to -1.060	Yes	*	
Mock Transfection vs. Wa GS1 Plasmid (VP1)	2.733	-0.1471 to 5.614	No	ns	
Mock Transfection vs. Wa GS2 Plasmid (VP2)	5.4	2.078 to 8.722	Yes	*	
Mock Transfection vs. Wa GS3 Plasmid (VP3)	-1.8	-5.756 to 2.156	No	ns	
Mock Transfection vs. Wa GS4 Plasmid (VP4)	3.433	-0.3687 to 7.235	No	ns	
Mock Transfection vs. Wa GS5 Plasmid (NSP1)	1.7	-3.185 to 6.585	No	ns	
Mock Transfection vs. Wa GS6 Plasmid (VP6)	-1.7	-2.207 to -1.193	Yes	**	
Mock Transfection vs. Wa GS7 Plasmid (NSP3)	-0.4667	-4.061 to 3.127	No	ns	
Mock Transfection vs. Wa GS8 Plasmid (NSP2)	-4.167	-7.262 to -	Yes	*	

		1.071			
Mock Transfection vs. Wa GS9 Plasmid (VP7)	0.4667	-3.959 to 4.892	No	ns	
Mock Transfection vs. Wa GS10 Plasmid (NSP4)	-1.333	-7.586 to 4.919	No	ns	
Mock Transfection vs. Wa GS11 Plasmid (NSP5/6)	3.733	-0.2014 to 7.668	No	ns	
Mock Transfection vs. All Wa Plasmids (GS1-11)	-1.767	-5.063 to 1.529	No	ns	
Mock Transfection vs. SA11 Alpha Plasmid (VP1 + NSP2 + NSP5/6)	-2.267	-3.040 to -1.493	Yes	**	
Mock Transfection vs. SA11 Beta Plasmid (VP2 + VP3)	3.033	-2.066 to 8.133	No	ns	
Mock Transfection vs. SA11 Gamma Plasmid (VP4 + NSP1 + VP6)	5.667	3.619 to 7.714	Yes	**	
Mock Transfection vs. SA11 Delta Plasmid (NSP4 + VP7 + NSP3)	-3.167	-4.795 to -1.538	Yes	*	
Mock Transfection vs. All SA11 Plasmids (Alpha-Delta)	5.4	2.361 to 8.439	Yes	*	

Test details	**Mean 1**	**Mean 2**	**Mean Diff.**	**SE of diff.**	**n1**
Mock Transfection vs. Cell Control	0.4333	0.3333	0.1	0.2	3
Mock Transfection vs. SA11 infection	0.4333	2.733	-2.3	0.05774	3
Mock Transfection vs. SA11 mRNA transcripts	0.4333	2.833	-2.4	0.1528	3
Mock Transfection vs. Wa GS1 Plasmid (VP1)	0.4333	-2.3	2.733	0.3283	3
Mock Transfection vs. Wa GS2 Plasmid (VP2)	0.4333	-4.967	5.4	0.3786	3
Mock Transfection vs. Wa GS3 Plasmid (VP3)	0.4333	2.233	-1.8	0.4509	3
Mock Transfection vs. Wa GS4 Plasmid (VP4)	0.4333	-3	3.433	0.4333	3
Mock Transfection vs. Wa GS5 Plasmid (NSP1)	0.4333	-1.267	1.7	0.5568	3
Mock Transfection vs. Wa GS6 Plasmid (VP6)	0.4333	2.133	-1.7	0.05774	3
Mock Transfection vs. Wa GS7 Plasmid (NSP3)	0.4333	0.9	-0.4667	0.4096	3
Mock Transfection vs. Wa GS8 Plasmid (NSP2)	0.4333	4.6	-4.167	0.3528	3
Mock Transfection vs. Wa GS9 Plasmid (VP7)	0.4333	-0.03333	0.4667	0.5044	3
Mock Transfection vs. Wa GS10 Plasmid (NSP4)	0.4333	1.767	-1.333	0.7126	3
Mock Transfection vs. Wa GS11 Plasmid (NSP5/6)	0.4333	-3.3	3.733	0.4485	3
Mock Transfection vs. All Wa Plasmids (GS1-11)	0.4333	2.2	-1.767	0.3756	3
Mock Transfection vs. SA11 Alpha Plasmid (VP1 + NSP2 + NSP5/6)	0.4333	2.7	-2.267	0.08819	3
Mock Transfection vs. SA11 Beta Plasmid (VP2 + VP3)	0.4333	-2.6	3.033	0.5812	3
Mock Transfection vs. SA11 Gamma Plasmid (VP4 + NSP1 + VP6)	0.4333	-5.233	5.667	0.2333	3
Mock Transfection vs. SA11 Delta Plasmid (NSP4 + VP7 + NSP3)	0.4333	3.6	-3.167	0.1856	3
Mock Transfection vs. All SA11 Plasmids (Alpha-Delta)	0.4333	-4.967	5.4	0.3464	3

Supplementary Table F11: RM one-way ANOVA of RIP1: ANOVA

	Data Set-A	Data Set-B	Data Set-C	Data Set-D	Data Set-E
Table Analyzed	RIP1				
Repeated measures ANOVA summary					
Assume sphericity?	No				
F	285.3				
P value	< 0.0001				
P value summary	****				
Statistically significant (P < 0.05)?	Yes				
Geisser-Greenhouse's epsilon	0.0923				
R square	0.993				
Was the matching effective?					
F	1.109				
P value	0.3399				
P value summary	ns				
Is there significant matching (P < 0.05)?	No				
R square	0.0003858				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	1215	20	60.77	F (1.846, 3.692) = 285.3	P < 0.0001
Individual (between rows)	0.4724	2	0.2362	F (2, 40) = 1.109	P = 0.3399
Residual (random)	8.521	40	0.213		
Total	1224	62			
Data summary					
Number of treatments (columns)	21				
Number of subjects (rows)	3				

Supplementary Table F12: RM one-way ANOVA of RIP1: Multiple comparisons

	Data Set-A	Data Set-B	Data Set-C	Data Set-D	Data Set-E
Number of families	1				
Number of comparisons per family	20				
Alpha	0.05				

Dunnett's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
Mock Transfection vs. Cell Control	0.4	-2.420 to 3.220	No	ns	0.8559
Mock Transfection vs. SA11 infection	-2.467	-3.521 to -1.412	Yes	**	0.0094
Mock Transfection vs. SA11 mRNA transcripts	-2.267	-6.069 to 1.535	No	ns	0.1325
Mock Transfection vs. Wa GS1 Plasmid (VP1)	2.7	-0.9529 to 6.353	No	ns	0.089
Mock Transfection vs. Wa GS2 Plasmid (VP2)	5.933	1.227 to 10.64	Yes	*	0.0319
Mock Transfection vs. Wa GS3 Plasmid (VP3)	-4.667	-9.481 to 0.1479	No	ns	0.0531
Mock Transfection vs. Wa GS4 Plasmid (VP4)	1.867	-4.508 to 8.241	No	ns	0.4243
Mock Transfection vs. Wa GS5 Plasmid (NSP1)	0.7333	-0.3212 to 1.788	No	ns	0.0998
Mock Transfection vs. Wa GS6 Plasmid (VP6)	-10.33	-14.07 to -6.599	Yes	**	0.0067
Mock Transfection vs. Wa GS7 Plasmid (NSP3)	-12.93	-18.93 to -6.932	Yes	*	0.0111
Mock Transfection vs. Wa GS8 Plasmid (NSP2)	-5.167	-8.423 to -1.910	Yes	*	0.0203
Mock Transfection vs. Wa GS9 Plasmid (VP7)	0.4	-5.442 to 6.242	No	ns	0.9962
Mock Transfection vs. Wa GS10 Plasmid (NSP4)	-4.233	-7.606 to -0.8604	Yes	*	0.0321
Mock Transfection vs. Wa GS11 Plasmid (NSP5/6)	-4.6	-6.921 to -2.279	Yes	*	0.0131
Mock Transfection vs. All Wa Plasmids (GS1-11)	-0.5333	-6.405 to 5.338	No	ns	0.9775
Mock Transfection vs. SA11 Alpha Plasmid (VP1 + NSP2 + NSP5/6)	-2.1	-3.440 to -0.7597	Yes	*	0.0208
Mock Transfection vs. SA11 Beta Plasmid (VP2 + VP3)	-1.033	-6.133 to 4.066	No	ns	0.6563
Mock Transfection vs. SA11 Gamma Plasmid (VP4 + NSP1 + VP6)	1.1	-1.720 to 3.920	No	ns	0.2758
Mock Transfection vs. SA11 Delta Plasmid (NSP4 + VP7 + NSP3)	-2.533	-5.032 to -0.03449	Yes	*	0.0487
Mock Transfection vs. All SA11 Plasmids (Alpha-Delta)	5.333	0.5454 to 10.12	Yes	*	0.0406

Test details	**Mean 1**	**Mean 2**	**Mean Diff.**	**SE of diff.**	**n1**
Mock Transfection vs. Cell Control	0.3667	-0.03333	0.4	0.3215	3
Mock Transfection vs. SA11 infection	0.3667	2.833	-2.467	0.1202	3
Mock Transfection vs. SA11 mRNA transcripts	0.3667	2.633	-2.267	0.4333	3
Mock Transfection vs. Wa GS1 Plasmid (VP1)	0.3667	-2.333	2.7	0.4163	3
Mock Transfection vs. Wa GS2 Plasmid (VP2)	0.3667	-5.567	5.933	0.5364	3
Mock Transfection vs. Wa GS3 Plasmid (VP3)	0.3667	5.033	-4.667	0.5487	3
Mock Transfection vs. Wa GS4 Plasmid (VP4)	0.3667	-1.5	1.867	0.7265	3
Mock Transfection vs. Wa GS5 Plasmid (NSP1)	0.3667	-0.3667	0.7333	0.1202	3
Mock Transfection vs. Wa GS6 Plasmid (VP6)	0.3667	10.7	-10.33	0.4256	3

Mock Transfection vs. Wa GS7 Plasmid (NSP3)	0.3667	13.3	-12.93	0.6839	3
Mock Transfection vs. Wa GS8 Plasmid (NSP2)	0.3667	5.533	-5.167	0.3712	3
Mock Transfection vs. Wa GS9 Plasmid (VP7)	0.3667	-0.03333	0.4	0.6658	3
Mock Transfection vs. Wa GS10 Plasmid (NSP4)	0.3667	4.6	-4.233	0.3844	3
Mock Transfection vs. Wa GS11 Plasmid (NSP5/6)	0.3667	4.967	-4.6	0.2646	3
Mock Transfection vs. All Wa Plasmids (GS1-11)	0.3667	0.9	-0.5333	0.6692	3
Mock Transfection vs. SA11 Alpha Plasmid (VP1 + NSP2 + NSP5/6)	0.3667	2.467	-2.1	0.1528	3
Mock Transfection vs. SA11 Beta Plasmid (VP2 + VP3)	0.3667	1.4	-1.033	0.5812	3
Mock Transfection vs. SA11 Gamma Plasmid (VP4 + NSP1 + VP6)	0.3667	-0.7333	1.1	0.3215	3
Mock Transfection vs. SA11 Delta Plasmid (NSP4 + VP7 + NSP3)	0.3667	2.9	-2.533	0.2848	3
Mock Transfection vs. All SA11 Plasmids (Alpha-Delta)	0.3667	-4.967	5.333	0.5457	3

APPENDIX G

SUBMITTED MANUSCRIPT

The work performed in chapter 6 (***section 6.3.4***) has been submitted as a short communication to the Journal of General Virology.

Rotavirus non-structural proteins NSP1, NSP2 and NSP5 suppress several innate immune responses in cells transfected with rotavirus (+)single-stranded RNAs

J. F. Wentzel[a], L. H. du Plessis[b], L. Mlera[a, c], H.G. O'Neill[d] and A. A. van Dijk[a]

[a] Biochemistry Division, North-West University, Potchefstroom, South Africa

[b] Centre of Excellence for Pharmaceutical Sciences (Pharmacen), North-West University, Potchefstroom, South Africa

[d] Department of Microbial, Biochemical and Food Biotechnology, University of the Free State, Bloemfontein, South Africa

Corresponding author: A. A. van Dijk,

Biochemistry Division, North-West University,

Private Bag X6001 Noordbrug, 2520 Potchefstroom, South Africa

Tel: +27 18 299 2317

Fax: +27 18 299 2316

E-mail: albie.vandijk@nwu.ac.za

[c] Present address: Laboratory of Virology, National Institutes of Allergy and Infectious Disease, National Institutes of Health, 903 South 4th Street, Hamilton, Montana 59840, USA

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Abstract

To date, studies in cell culture involving rotavirus (RV) (+)ssRNA transcripts have been hampered by the aggressive innate immune response (IIR) they elicit by inducing expression of cytokines of the interferon (IFN) family and IFN-stimulated genes. In this study, type I and III IFN responses were also detected following the transfection of SA11 (+)ssRNA transcripts. Expression of VP1, NSP2, NSP5 and VP6 from transfected, *in vitro* transcribed SA11 (+)ssRNA transcripts was confirmed with immunodetection assays in MA104 and HEK293H cells. This is the first proof that *in vitro* transcribed RV (+)ssRNAs can be translated in cells. Transfecting plasmids expressing NSP1 or NSP2 before transfecting RV (+)ssRNAs reduced the expression of type I IFN and CXCL10, whereas prior co-expression of NSP2 and NSP5 (viroplasm-like structure) significantly suppressed type I and III IFN responses. The finding that co-expression of NSP2 and NSP5 suppressed the IIR in transfected cells suggests a possible novel way of RV immune evasion.

Rotaviruses are the leading cause of acute, dehydrating gastroenteritis among infants and young children under the age of five, causing approximately 1 200 deaths globally each day (Tate *et al.*, 2012). Most viral infections lead to the stimulation of a complex cascade of host cell signalling pathways that are required for mounting an effective antiviral response. The innate immune system is the cell's first line of defence against viruses by suppressing viral replication and precedes the adaptive immune response (Samuel, 2001). The secretion of cytokines belonging to the interferon (IFN) family (type I and III IFN) play an important part in this innate immune response (IIR) by activating the expression of IFN-stimulated genes (ISGs) (Randall & Goodbourn, 2008; Takeuchi & Akira, 2009). ISGs directly inhibit viral replication and induce an antiviral state with the intention of annihilating the virus-infected cells before the infectious agent can complete its replication cycle and infect other cells (Edinger & Thompson, 2004; Levy *et al.*, 2011; Stetson & Medzhitov, 2006).

Some viruses have evolved specific immune evasion strategies to neutralize and exploit these immune responses to the benefit of their own replication cycle. The simian SA11 and bovine UKtc strains of RV, in particular, suppress the type I IFN response by NSP1 (non-structural protein 1, encoded by genome segment 5). The NSP1 of these strains is thought to be an E3 ubiquitin ligase which is able to bind to various IFN regulation factors (IRF3/5/7) and mark them for proteasomal degradation (Barro & Patton, 2007; Graff *et al.*, 2007). On the other hand, NSP1 of the porcine OSU strain induces the degradation of b-TrCP, an essential protein in the NF-κB pathway, but is unable to degrade IRF3 degradation (Arnold & Patton, 2011). Apart from IRF degradation, there is evidence that NSP1 down-regulates the RNA sensitive retinoic acid-induced gene (RIG-I) (Qin *et al.*, 2011). The viral suppression of the induction of the IFN response by NSP1 is still not fully understood.

To date, studies involving RV transcripts in cultured cells have been hampered by the aggressive innate immune responses to the viral mRNA (Melera, 2013; Uzri & Greenberg, 2013). This IIR to RV transcripts is still not fully understood. The main aim of this study was to determine the potential of overexpressed RV proteins to supress the interferon response elicited by RV transcripts.

cDNA cassettes containing the RV WaCS (Wentzel *et al.*, 2013) genome segments encoding NSP1, NSP2, NSP5, VP1 and VP6, respectively, were synthesized by GenScript (Piscatawy, NY, USA) (***see Supplementary Figure 1 for cassette composition***). Rhesus monkey kidney cells (MA104) and human embryonic kidney cells (HEK293H) were used to evaluate the effect of expressed RV proteins on cultured cells following the transfection of RV transcripts. A dual transfection strategy was followed. For the first transfection, 4 µg of each plasmid containing the individual expression cassette encoding the specific RV Wa proteins (NSP1, NSP2, NSP5, VP1 and VP6) was transfected. A second transfection of *in vitro* derived RV SA11 transcripts (0.5 µg) was performed 24 hours after the first plasmid transfection (***see supplementary data for complete materials and methods***). Mass cell death was observed within 24 hours in cells transfected only with the *in vitro* derived RV transcripts (***Supplementary Table 1***). Transfecting plasmids expressing either NSP1, NSP2 or a combination of NSP2 and NSP5 24 hours before transfecting RV transcripts, reduced and delayed cell death. The expression of VP1 and VP6 had no effect on delaying the cell death caused by the RV transcripts. Expression of RV VP1, NSP2 and NSP5 derived from transcripts and recombinant plasmids was confirmed by immunofluorescence (***Figure 1A***). The expression of VP6, also from transfected transcripts and recombinant plasmid was determined by streptavidin-biotin immunoenzymatic antigen detection (***Figure 1B***). This

result is in contrast to a recent report (Richards *et al.*, 2013) and indicates, for the first time, that transfected *in vitro* derived transcripts can be translated in cultured cells. Longer incubation times with a less toxic transfection reagent, suitable cell culture system and correct concentration of the RV mRNA seem to be crucial for RV transcript translation *in vitro*. In general, the intracellular expression from transfected plasmids was much higher than that from transfected RV transcripts. Since no commercially or other antibody to NSP1 was available to us, expression thereof could not be verified.

In order to examine the expression of specific innate response cytokines, quantitative real time PCR (qRT-PCR) amplification of cDNA was performed using a TaqMan Universal PCR Master Mix buffer and commercial probes. For qRT-PCR experiments, HEK293H cells were transfected with plasmids (4 µg) containing either the genome segment encoding NSP1, NSP2, NSP5, VP1, VP6 or a combination of NSP2 and NSP5 (viroplasm-like structure) (Fabbretti *et al.*, 1999). HEK293H cells were chosen for the qRT-PCR due to their high transfection efficiency and compatibility with the commercially available human gene expression probes. After 24 hours, a second transfection of 0.5 µg RV SA11 transcripts was performed. Cells were harvested after 20 hours following the second transfection, total RNA was isolated and cDNA was synthesized. The expression levels of the selected cytokines were subsequently compared using qRT-PCR with TaqMan® Gene Expression Assays (Applied Biosystems). Expression of type I interferons (IFN-α1 and IFN-1β), type III interferon (IFN-λ1) and interferon γ-induced protein 10 (CXCL10) were examined. Analyses were done on a Applied Biosystem 7500 thermo cycler and the relative quantity of the RNA of a specific cytokine was normalised to the 18S rRNA internal standard (Applied Biosystems) using the

$2^{-\Delta\Delta C}{}_T$ method. When comparing cytokine expression levels, the differences in C_T values were taken as the $\log_2$ of the relative starting concentrations of the 18S rRNA internal control (Kuchipudi *et al.*, 2012). In order to measure the IFN response of a normal RV infection, HEK293H cells were infected with trypsin activated RV SA11. The TaqMan Gene Expression assays revealed that transfection with the plasmid encoding NSP1 24 hours before transfecting RV SA11 mRNA reduced the expression of type I IFNs (IFN-1α (***Figure 2A***), IFN-1β (***Figure 2B***)) and expression of the interferon induced cytokine (CXCL10) (***Figure 2D)***. RV NSP1 is a strain specific mRNA binding protein and the only RV protein known to be directly concerned with the evasion of the IIR of the host cell (Bagchi *et al.*, 2010; Feng *et al.*, 2009). The NSP1 of RV SA11 and UKtc has been shown to bind IRF3, 5 and 7 in order to facilitate their breakdown (Barro & Patton, 2007). Expressing plasmid-derived NSP1 of several RV strains in cell culture has been reported to counter the innate response to normal RV infections (Arnold & Patton, 2011). Our results indicate that expressing Wa NSP1 can also suppress the IFN-1α and IFN-1β responses in cells transfected with RV mRNA (***Figure 2A and 2B***). However, RV Wa NSP1 did not reduce expression of the type III IFN, IFN-λ1 (***Figure 2C***). Type III IFNs (IFNλ, interleukin 28/29 or IL28/29) are the latest identified members of an established IFN family (Ank *et al.*, 2006; Kotenko *et al.*, 2003) and their action is exerted through a unique receptor complex (Kotenko *et al.*, 2003; Zhou *et al.*, 2007).

Expressing NSP2 before transfection of RV SA11 mRNA also reduced the type I IFN reaction to viral transcripts (***Figure 2A and 2B***). Apart from being an integral part of the viroplasm, several functions have been suggested for NSP2, including being involved in genome

packaging and replication (Estes & Kapikian, 2007). The only other report of a reduction in the type I IFN production associated with expression of NSP2 (from RRV and SA11) is in plasmacytoid dendritic cells (Deal *et al.*, 2010), but neither the mechanism, nor function, of this suppression is known.

Interestingly, the most profound suppression of the IFN response resulted from the co-expression of NSP2 and NSP5 (***Figure 2A-D***). When co-expressed, NSP2 and NSP5 form viroplasm-like structures (Fabbretti *et al.*, 1999), similar to the cytoplasmic inclusion bodies (viroplasms) found in normal RV infected cells. Viroplasm-like structures have no known direct influence on the host's innate immune system. Due to the association of viroplasms with the viral proteins linked to replication, synthesis of minus-sense strand ssRNA (to form genomic dsRNA) is thought to take place in these inclusion bodies, where the ssRNA is isolated from the host's innate immune system (Contin *et al.*, 2010; Fabbretti *et al.*, 1999). It can be speculated that the transfected RV (+)ssRNAs, mRNA, may be shielded from the innate immune system by the plasmid-mediated NSP2/5 expressed viroplasm-like structures. Although cells first transfected with the expression plasmids generating NSP2 and NSP5 showed lower cytokines expression levels at 24 hours post transfection, onset of mass cell death was observed 40 hours after the transfection of mRNA transcripts (***Supplementary Table 1***). This may suggest that there are other IIR suppression mechanisms in addition to the IFN responses investigated in this study.

Transfection of plasmids encoding NSP5, VP1 and VP6 prior to RV transcript transfection did not influence the expression of IFNs and exhibited similar cell death patterns and levels of

cytokine expression as seen in cells only transfected with RV transcripts (***Supplementary Table 1***, ***Figure 2***). Transfecting the individual plasmids expressing NSP1, NSP2, NSP5, VP1, VP6 or the combination of NSP2/5 without a second transfection with RV transcripts did not induce significant expression of any of the tested cytokines (results not shown).

To determine if the IIR elicited by RV (+)ssRNA is rotavirus specific, other *in vitro* derived transcripts were tested to determine if they elicit the same potent innate response as RV transcripts. The mRNAs were prepared from plasmids containing the ORF of a human metabolic enzyme, human glycine N-acyl tranferase (GLYAT), and segments S and M of another RNA virus, Rift Valley fever virus (RVFV), by *in vitro* transcription and then transfected. Neither GLYAT mRNA nor mRNA of segments S and M of RVFV significantly induced the expression of any of the tested cytokines (***Figure 2E***).

It is known that RV infection leads to the activation of RNA-sensitive retinoic acid-induced gene (RIG-I) and melanoma differentiation associated gene 5 (MDA-5) (Sen *et al.*, 2011). Using RIG-I and MDA5 wild type and knockout murine embryonic fibroblast cell cultures, it has recently been shown that the RIG-I and MDA5 are also activated by RV transcripts (Uzri & Greenberg, 2013). Therefore, the effect of expressed RV proteins on the expression of RIG-I and MDA5 was also investigated in HEK293H cells by flow cytometry (FACSCalibur, BD Biosciences). The same level of RIG-I and MDA5 expression was observed in cells into which expression plasmids for NSP1 or NSP2 and NSP5 were transfected, prior to transfection of RV SA11 transcripts, as in cells only transfected with RV RNA transcripts (***Figure 3***). It was previously shown that NSP1 of RV OSU and SA11 can supress the expression of RIG-I (Qin *et*

al., 2011), but this does not seem to be the case for RV Wa NSP1 in the innate response elicited by RV mRNA.

In this report, plasmids encoding human RV Wa NSP1, NSP2 or a combination of NSP2 and NSP5 were used to suppress the IFN response provoked by SA11 RV (+)ssRNA transcripts. In normal RV infections, NSP1 triggers the degradation of several type I IFN transcription factors. Plasmid derived NSP1 also suppressed expression of type I IFN in HEK293H cells induced by RV mRNA, but not type III IFN. The non-structural protein, NSP2, marginally reduced the expression of type I and III IFNs. NSP2 and NSP5 form the viroplasm-like structure in which most of the RV replication is thought to occur (Estes & Kapikian, 2007). Co-expression of NSP2 and NSP5 plasmids dramatically reduced cell death and suppressed both type I and III IFN systems after subsequent RV SA11 mRNA transfection. Further investigation is needed to determine if the co-expression of NSP2 and NSP5 can directly inhibit the IIR or if it is only shielding the RV transcript from the innate immune system through the formation of the viroplasm-like structure. The data presented here suggest that RV may use other mechanisms in addition to the degradation of IRFs by NSP1 to circumvent the cell's innate immune response.

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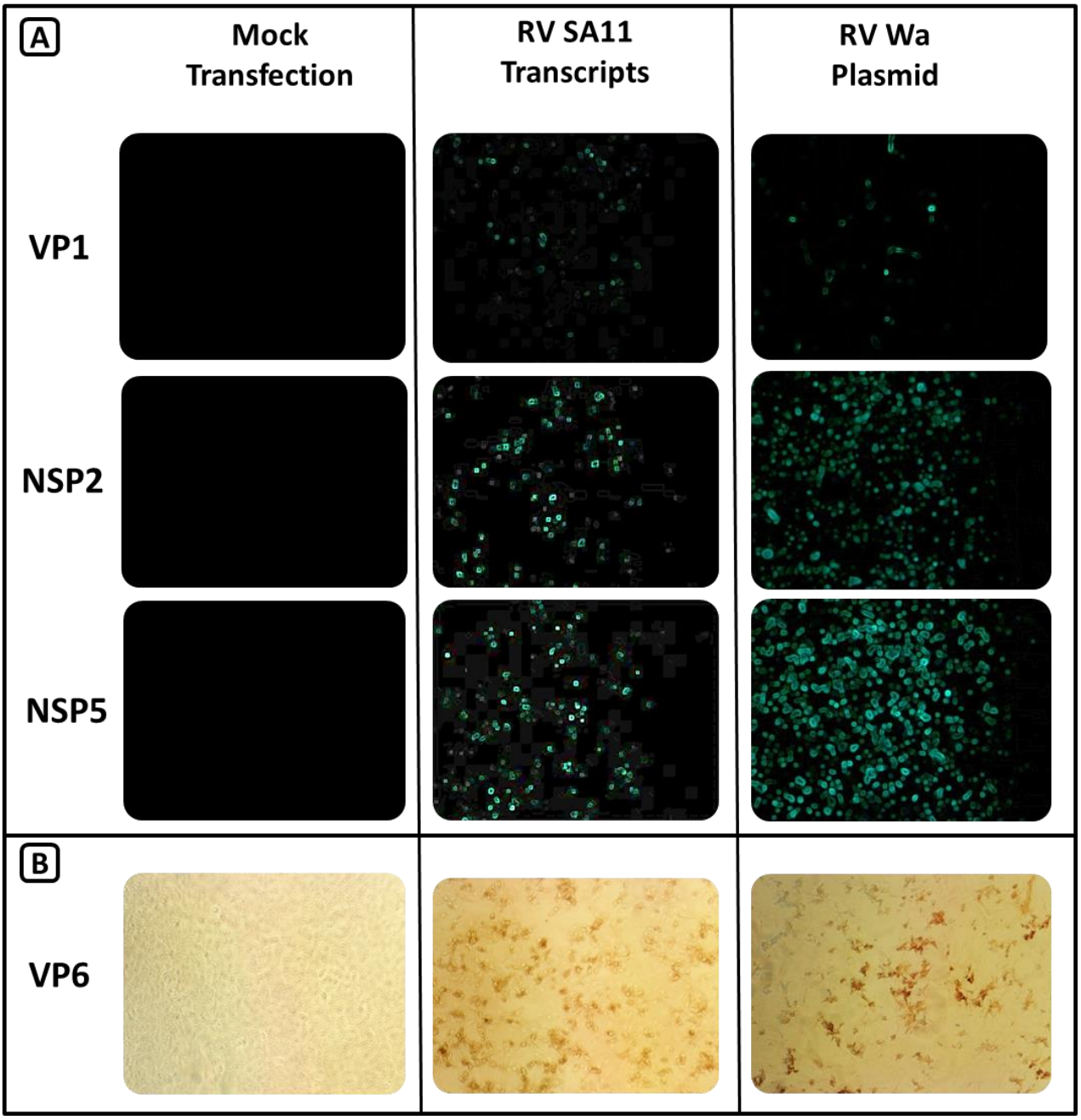


Figure 1: **Immunological detection of rotavirus protein expression. Cells were immunostained with antibodies showing the expression of rotavirus proteins in HEK293H cells following the transfection of rotavirus Wa plasmids or *in vitro* derived rotavirus SA11 transcripts.** The expression of rotavirus (A) VP1, NSP2 and NSP5 were determined by immunofluorescence. Primary guinea pig antibodies to VP1, NSP2 d NSP5 were used. The expression rotavirus (B) VP6 was determined by streptavidin-biotin immunoenzymatic antigen detection (rabbit specific HRP/DAB detection kit from Abcam).

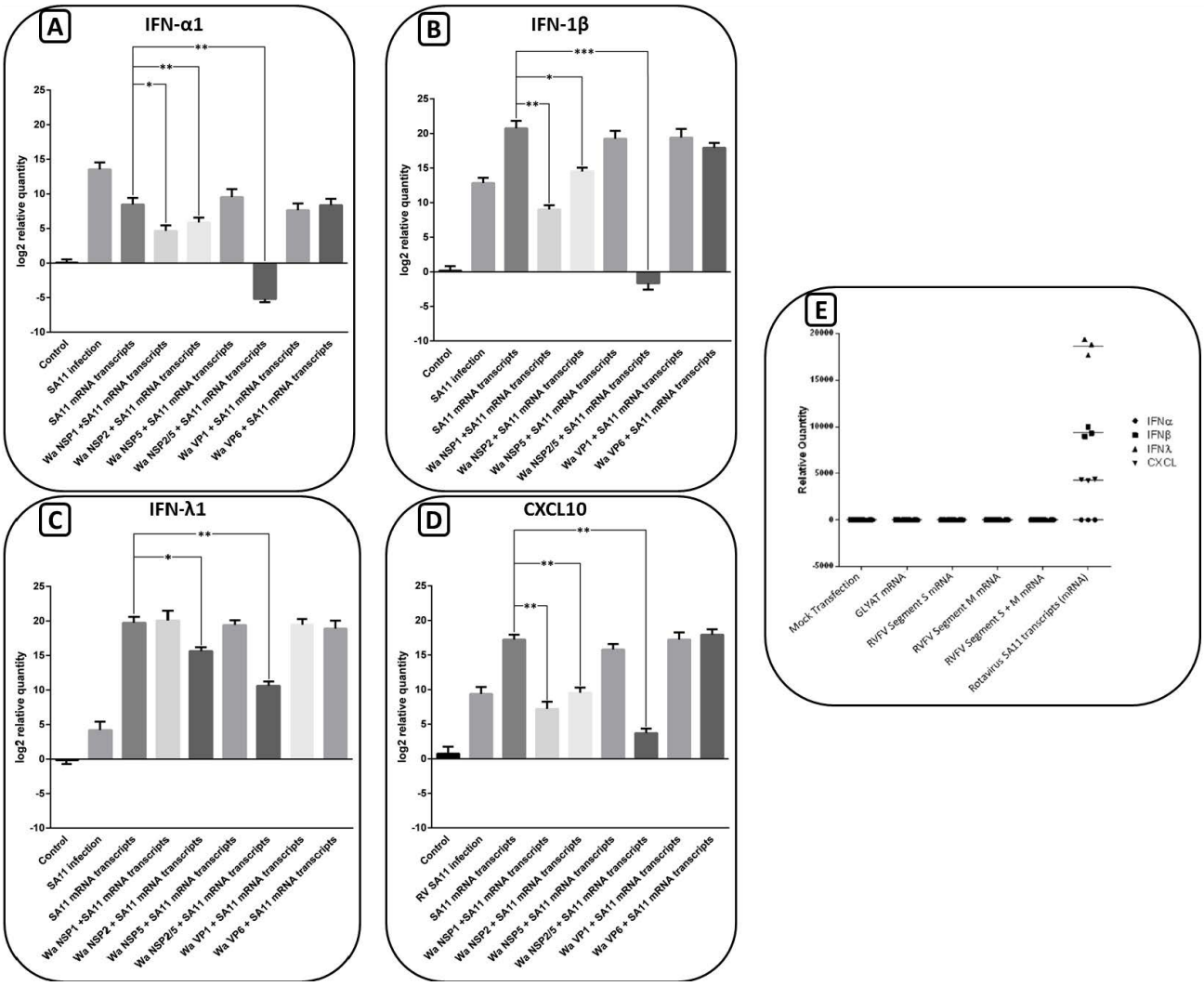
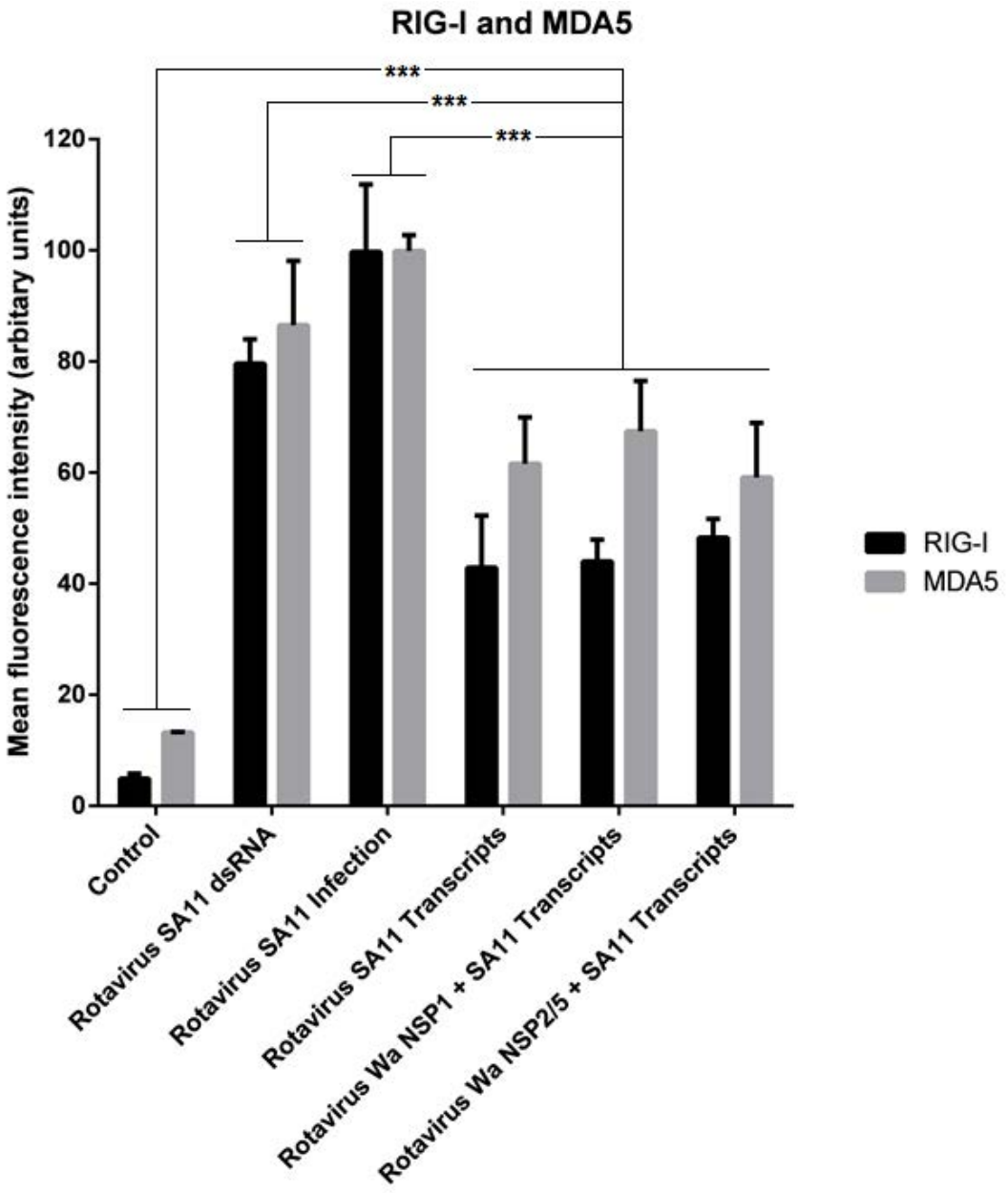


Figure 2: (A) **The log2 relative quantities of cytokine-encoding mRNA expression induced in HEK293H cells 24 hours after transfection of rotavirus SA11 transcripts.** Graphs **A–D** indicate the effect of expressing different rotavirus Wa proteins on the induction of IFNα1 (A), IFN-1β (B), IFN-λ1 (C) and CXCL10 (D) in HEK293H cells by rotavirus SA11 transcripts. Statistically significant expression differences are indicated by stars (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$). ***(B)*** **Comparison of the relative quantities of the expression of various cytokines induced in HEK293H cells by *in vitro* derived GLYAT-, RVFV- and rotavirus transcripts.** Expression of IFN-α 1, IFN-1β, IFN-λ1 and CXCL10 was determined by qRT-PCR after the transfection of in vitro-derived transcripts encoding glycine N-acyl transferase (GLYAT), segments S and M of Rift Valley fever virus (RVFV) and rotavirus. All values are the arithmetic mean and standard deviation of at least three experimental replicates done in triplicate.



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334 ***Figure 3:*** **Comparison of flow cytometry-detected expression of RIG-I and MDA5 induced**
335 **in HEK293H cells by rotavirus transcripts and plasmids**. HEK293H cells were transfected
336 with plasmids expressing different rotavirus Wa proteins before transfection with rotavirus
337 SA11 transcripts. Rotavirus SA11 dsRNA was used as a positive control for the expression of
338 RIG-I and MDA5. The expression of RIG-I and MDA5 in a normal rotavirus SA11 infection
339 were also examined. Statistically significant expression differences are indicated by stars
340 (*** $P \leq 0.001$).

341

342

Supplementary materials and methods

Cells

Human embryonic kidney cells (HEK 293H) and rhesus monkey kidney cells (MA104) were maintained in DMEM (Hyclone) containing 1% penicillin/streptomycin/amphotericin (Lonza) and 1% non-essential amino acids (Gibco). The medium was fortified with 10% foetal bovine serum (Hyclone). Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2.

Preparation of transcriptionally active rotavirus SA11 double-layered particles

Rotavirus SA11 (G3P[2]) was propagated in bulk at a MOI of ~0.3 by incubating rotavirus-infected MA104 cells in DMEM containing 1 µg/ml porcine trypsin IX (Sigma) and supplemented with 1% non-essential amino acids (Gibco) and 1% penicillin/streptomycin/amphotericin (Lonza). Infected cultures were harvested when the cytopathic effect (CPE) reached approximately 80%. Cells were harvested by freeze-thowing. The cellular components were separated from the culture medium by centrifugation at 800 xg for 5 minutes at 4 °C. The remaining supernatant was ultra-centrifuged at 150 000 xg, using a TH641 rotor in a Sorvall ultracentrifuge at 4 °C, for 1 hour. The two pellets were pooled and resuspended in 8 ml of 10 mM Tris/HCl buffered saline (TBS), pH 7.5. After resuspension, 3.2 ml Vertrel® (DuPont) was added and the mixture homogenized. The homogenates were centrifuged at 400 xg at 4 °C for 5 minutes and pooled. The rotavirus outer capsid (VP4 and VP7) proteins were removed with 10 mM EDTA and incubated at 37 °C for 1 hour (Estes *et al.*, 1979) followed by pelleting the double layered particles (DLPs) by ultra-centrifugation in a TH641 rotor at 165 000 xg for 1.5 hours. The resulting pellet was resuspended in TBS and purified by CsCl (Sigma) gradient ultra-centrifugation at 200 000 xg

for 16 hours at 15 °C with a TH641 rotor. The band containing the DLPs was extracted using a syringe and the remaining CsCl removed by dialysis in TBS overnight.

In vitro transcription of the rotavirus SA11 genome segments using double-layered particles

For the *in vitro* transcription of rotavirus SA11 genome segments, a transcription mixture was prepared containing 100 mM Tris/HCl (pH 8.0), 50 mM sodium acetate, 10 mM magnesium acetate, 1 mM DTT (Roche), 5 mM rATP (Promega), rCTP, rGTP, rUTP, at 2.5 mM each, 1 mM SAM (Sigma), 6% PEG6000 and 0.4 U/µl RNasin® Plus (Promega) (Mason *et al.*, 1980). The transcription reaction was performed at 40 °C for 6 h. Total RNA was extracted from the transcription reaction using TRI-reagent LS (Molecular Research Centre), following a standard RNA extraction protocol (Jere *et al.*, 2011). Viral genomic dsRNA was removed by precipitating ssRNA with 2 M LiCl at 4 °C for 16 hours followed by centrifugation at 15 000 xg for 30 minutes at 4 °C. The mRNA pellet was washed with 70% ethanol and dissolved in nuclease-free water (Promega) containing 0.5 U/µl RNasin® Plus.

Construction of expression cassettes containing rotavirus genome segments

cDNA cassettes containing the rotavirus Wa genome segments encoding NSP1, NSP2, NSP5, VP1 and VP6 were designed. Each genome segment insert was engineered to contain a T7 promoter region, HDV ribozyme region and a T7 termination region (***Supplementary Figure 1***) (Kobayashi *et al.*, 2007). Every genome segment insert is flanked by a unique restriction enzyme site. Rotavirus Wa expression cassettes were synthesized by GenScript (USA) and cloned in pUC57 plasmids (Thermo Scientific).

Determination of the effect of rotavirus SA11 transcripts on cell viability

To determine cytopathic effect of rotavirus transcripts on mammalian cells, HEK 293H and MA104 were transfected with 0.5 µg *in vitro* derived rotavirus SA11 transcripts using the X-tremeGENE HP (Roche) transfection reagent as prescribed by the manufacturer. The cells were visually monitored over a 48 hour period. In addition, cells were also transfected with 4 µg of pUC57 plasmids encoding NSP1, NSP2, NSP5, VP1, VP6 or a combination of NSP2 and NSP5 (viroplasm-like structure). After 24 hours, a second transfection of 0.5 µg rotavirus SA11 transcripts was performed and cell viability was visually monitored over a 48 hour period.

Determining the expression of certain rotavirus SA11 transcript and plasmid-derived rotavirus Wa proteins

About 16 hours before the first transfection, HEK 293H and MA104 cells were seeded in 24-well plates (Nunc^{TM}). At approximately 80–90% confluence, the cells were transfected using the XtremeGENE HP transfection reagent (Roche) as prescribed by the manufacturer. The T7 polymerase was provided by a recombinant fowlpox virus system as described by previously by Britton and co-workers and Skinner *et al*. (Britton *et al.*, 1996; Skinner *et al.*, 2005). Each well of the 24-well plate was transfected with 0.5 µg rotavirus SA11 transcripts or 4 µg of pUC57 plasmid DNA encoding VP1, VP6, NSP2 or NSP5 using the X-tremeGENE HP transfection reagent. The transfection mixture was left on the cells for the duration of the experiment. After 24 hours, cells were fixed using 4% paraformaldehyde followed by permeabilization with 0.25% Triton X-100 (Merck). The rabbit specific HRP/DAB detection kit (Abcam) was used to prepare cells for rotavirus VP6 immunostaining. The primary antibody

(goat polyclonal to rotavirus NCDV (Biotin)) was diluted 1:200 in PBS and incubated at room temperature for 2 hours. The secondary antibody (donkey polyclonal secondary antibody to goat IgG - H&L (HRP)) was diluted 1:400 in PBS and incubated for 1 hour at room temperature. To visualize the secondary antibody, DAB chromagen solution (Abcam) was mixed with 1 ml of the DAB substrate. The solution was incubated in a darkened space for 10 min at room temperature. The immunostained cells were then visualised with an Eclipse TE2000-S microscope (Nikon) and images were captured using the NIS-Elements (2.30) software (Nikon).

The expression of rotavirus VP1, NSP2 and NSP5 were determined by immunofluorescence microscopy. Primary guinea pig antibodies to VP1 and NSP2 were kindly donated by John Patton (NIH, USA) and primary guinea pig antibodies to NSP5 were a generous gift from Francesca Arnoldi and Oscar Burrone (ICGEB, Italy). The primary antibodies were diluted 1:200 in PBS and incubated at room temperature for 2 hours. A goat pAB to guinea pig (Abcam) secondary antibody was diluted 1:200 in PBS and incubated for 1 hour at room temperature. The immunofluorescence cells were also visualised with an Eclipse TE2000-S microscope (Nikon) and images were captured using the NIS-Elements (2.30) software (Nikon) (***Figure 1***). GFP was used as an expression control. Unfortunately we were unable to obtain antibodies to test for the expression of NSP1.

In vitro transcription of GLYAT and RVFV mRNA from plasmids

The cDNA of glycine N-acyl transferase (GLYAT) and the S- and M segments of Rift Valley fever virus (RVFV) were used as templates for *in vitro* transcription. Plasmids containing the S- and M segments of RVFV were a kind gift from Prof. Christiaan Potgieter (Deltamune, South Africa) and GLYAT containing plasmids were a donation from Dr. Chris Badenhorst

(NWU, South Africa). These plasmids were linearised with the appropriate restriction enzymes and used as a template for transcription. The DNA was then purified using the Qiagen Gel Extraction Kit (Qiagen) in accordance with the manufacturer's instructions. The purity and concentration of the excised DNA was determined using a NanoDrop® 1000 spectrophotometer. The mMESSAGE mMESSENGER® T7 Ultra kit (Ambion®) was used for the *in vitro* transcription of the linearised cDNA as prescribed by the manufacturer. Genome segments were transcribed individually in a 20 µl reaction. The *in vitro* transcription reaction was made up of T7 NTP/ARCA mixture (1X), T7 reaction buffer (5X) and T7 enzyme mix(0.5X) and 1µg of DNA template. After the removal of the DNA template with DNase (Ambion®), the transcripts were purified using a MEGAclear™ RNA purification kit (Ambion®) according to the manufacturer's instructions. The capping efficacy of the mMESSAGE mMACHINE T7 Ultra kit is between 70- 80%. Additional transcript capping was performed by the ScriptCap™ m7G capping system (Epicentre Biotechnologies). The capping reaction was done in a total volume of 20 µl and contained 1 µg of transcripts, capping buffer (1X), 1 mM GTP, 0.01 mM S-adenosyl methionine (SAM), 2 U/µl RNase inhibitor and 10 U capping enzyme. This additional capping reaction was performed at 37°C for 1 hour. After the capping procedure the transcripts were again purified with the MEGAclear™ kit (Ambion). The purity and concentration of the RNA was determined using a NanoDrop® 1000 spectrophotometer.

Determination of the expression of different cytokines

For quantitative real-time PCR (qRT-PCR) experiments, HEK 293H cells were transfected with pUC57 plasmids (4 µg) containing either the NSP1, NSP2, NSP5, VP1, VP6 or a combination

458 of NSP2 and NSP5 (viroplasm-like structure) genome segments using the X-tremeGENE HP
459 (Roche) transfection reagent and incubate at 37°C. After 24 hours, a second transfection of
460 0.5 µg *in vitro* derived rotavirus SA11 transcripts was performed. Cells were harvested 20
461 hours after the second transfection and total RNA was isolated. cDNA were synthesized
462 using the High Capacity RNA-to-cDNA master mix (Life Technologies) as prescribed by the
463 manufacturer. Comparisons were done by determining the expression levels of selected
464 cytokines using qRT-PCR with TaqMan® Gene Expression Assays (Applied Biosystems). Type I
465 interferons (IFN-α1) (assay ID: Hs00256882_s1), (IFN-1β) (Hs00277188_sl), type III
466 interferon (IFN-λ1) (Hs00601677_gl) and Interferon gamma-induced protein 10 (CXCL10)
467 (Hs00171042_ml) were chosen to be examined with qRT-PCR. A mock transfection was used
468 as a control. Analysis were done on a Applied Biosystem 7500 thermo cycler and relative
469 quantities of specific cytokine's RNA were normalised to *18S* mRNA (Applied Biosystems)
470 using the $2^{-\Delta\Delta C}{}_{T}$ method (Kuchipudi *et al.*, 2012). When comparing cytokine expression
471 levels, the differences in C_T values are the $\log_2$ of the relative starting concentrations of the
472 18S internal standard control. In order to gauge the IFN response of a normal rotavirus
473 infection, HEK 293H cells were infected with trypsin activated rotavirus SA11. HEK 293H cells
474 were infected with serial dilutions of RV SA11 and the expression of VP6 was compared to
475 that of 0.5 µg of RV SA11 transcripts. The multiplicity of infection was adapted to match the
476 expression levels of 0.5 µg of SA11 transcripts.
477
478 **Detection of the expression of RIG-I and MDA5 by flow cytometry**
479 For flow cytometry, HEK 293H cells were transfected with pUC57 (Thermo Scientific)
480 plasmids (4 µg) containing either the NSP1, NSP2, NSP5, VP1, VP6 or a combination of NSP2
481 and NSP5 (viroplasm-like structure) genome segments as described earlier. After 24 hours, a

second transfection of 0.5 µg *in vitro* derived RV SA11 transcripts was performed. The expression of RIG-I and MDA5 was determined by flow cytometry (FACSCalibur, BD Biosciences). Cells were fixed with methanol (0.1 %), treated with the primary antibody (anti-MDA5 or rabbit anti-RIG-I primary antibodies (Santa Cruz)) and thereafter the FITC (MDA5) (Santa Cruz) or PE (RIG-I) (Santa Cruz) secondary antibodies. Data were analysed by FCSExpress (version 4, De Novo Software).

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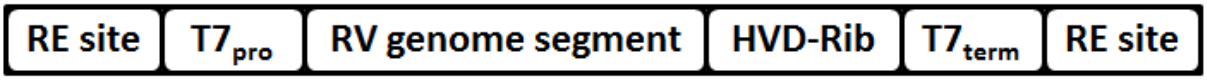

517 ***Supplementary Figure 1:*** **Rotavirus expression cassette design.** Each rotavirus genome
518 segment was placed under control of a T7 promoter region, followed by a HDV ribozyme
519 region and a T7 termination region.
520
521

Supplementary Table1: **Effect of expressed rotavirus proteins on the viability of MA104 and HEK 293 cells after transfection of *in vitro* derived rotavirus SA11 transcripts.**

Cell Type	Control[a]				SA11 infection[b]				SA11 Transcripts[c]				NSP1 + SA11 Transcripts[d]				NSP2 + SA11 Transcripts[d]				NSP5 + SA11 Transcripts[d]				NSP2/5 + SA11 Transcripts[d]				VP1 + SA11 Transcripts[d]				VP6 + SA11 Transcripts[d]			
Time after RV transcript transfection	12 h	24 h	36 h	48 h	12 h	24 h	36 h	48 h	12 h	24 h	36 h	48 h	12 h	24 h	36 h	48 h	12 h	24 h	36 h	48 h	12 h	24 h	36 h	48 h	12 h	24 h	36 h	48 h	12 h	24 h	36 h	48 h	12 h	24 h	36 h	48 h
HEK293H	-	-	-	+	+	++	++	+++	++	+++	++++	++++	+	++	+++	++++	+	++	+++	++++	++	+++	++++	++++	+	++	+++	++++	++	+++	++++	++++	++	+++	++++	++++
MA104	-	-	-	-	-	++	+++	+++	++	+++	++++	++++	+	++	+++	++++	+	++	+++	++++	++	+++	++++	++++	+	+	++	++++	+	+++	++++	++++	++	+++	++++	++++

[a] A mock transfection was used as a control. Experiments were carried out in duplicate. Representative results are presented of 3 repeat experiments.

[b] Cells were infected with a MOI of about 0.1 of rotavirus SA11 that gave comparable expression of VP6 as 0.5 µg of SA11 *in vitro* derived transcripts

[c] 0.5 µg of SA11 *in vitro* derived transcripts were used for transfection per well of a 6-well plate

[d] 4 µg of each plasmid containing rotavirus Wa genome segments encoding NSP1, NSP2, NSP5/6, VP1 and VP6, respectively, was used for transfection per well of a 6-well plate 24 hours before transfecting rotavirus transcripts. Time indicated is hours after RV transcript transfection.

- indicates no/very little cell death, + indicates 10-20% cell death, ++ indicates 20-30% cell death, +++ indicates 30-50% cell death and ++++ indicates mass cell death (70-100%)