Investigating the importance of co-expressed rotavirus proteins in the development of a selection-free rotavirus reverse genetics system

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Ek wou so graag ‘n liggie sien…

-Totius

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ABBREVIATIONS

µg:	Micro gram
µl:	Micro litre
^{o}C:	Degrees Celsius
aa:	Amino acid
ATP:	Adenosine triphosphate
BCA:	Bicinchoninic acid
bp:	Base pairs
cfu:	Colony forming units
CPE:	Cytopathic effect
CsCl:	Caesium chloride
Da:	Dalton
ddH2O	Double-distilled water
DNA:	Deoxyribonucleic acid
DLP:	Double-layered particle
DPI:	Days post infection
dsRNA:	Double-stranded ribonucleic acid
EB:	Elution buffer
EDTA:	Ethylene-diamine-tetra-acetic acid
ELISA:	Enzyme-linked immunosorbent assays
EM:	Electron microscope
ER:	Endoplasmic reticulum
FBS:	Foetal bovine serum
FDA:	Food and Drug Administration
GSK:	GlaxoSmithKline
HIV:	Human immunodeficiency virus

HPI:	Hours post infection
H:	Hour
HSC70:	Heat-shock cognate 70 proteins
IgA:	Immunoglobulin A
IgG:	Immunoglobulin B
IgM:	Immunoglobulin M
LB:	Lysogeny broth
kDa:	Kilo Dalton
MA104:	African green monkey kidney cell line
MOI:	Multiplicity of infection
ml:	Millilitre
NIH:	National Institutes of Health
NTPase:	Nucleosidetriphosphatase
NWU:	North-West University
NSP:	Non-structural protein
OD:	Optical density
ORF:	Open reading frame
PAGE:	Polyacrylamide gel electrophoresis
PBS:	Phosphate-buffered saline
PCR:	Polymerase chain reaction
pfu:	Plaque forming units
PLC:	Phospholipase C
RdRp:	RNA-dependent RNA polymerase
RE:	Restriction endonuclease
RNA:	Ribonucleic acid
RPM:	Revolutions per minute
RT-PCR:	Real time polymerase chain reaction
siRNA:	Small interfering ribonucleic acid

SOC:	Super optimal broth with catabolite repression
ssRNA:	Single-stranded ribonucleic acid
SDS-PAGE:	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
siRNA:	Small interfering RNA
SLP:	Single-layered particle
TLP:	Triple-layered particle
TOI:	Time of infection
UTR:	Untranslated terminal region
V:	Volts
VP:	Structural viral protein
WHO:	World Health Organisation

SUMMARY

Reverse genetics is an innovative molecular biology tool that enables the manipulation of viral genomes at the cDNA level in order to generate particular mutants or artificial viruses. The reverse genetics system for the influenza virus is arguably one of the best illustrations of the potential power of this technology. This reverse genetics system is the basis for the ability to regularly adapt influenza vaccines strains. Today, reverse genetic systems have been developed for many animal RNA viruses. Selection-free reverse genetics systems have been developed for the members of the *Reoviridae* family including, African horsesickness virus, bluetongue virus and orthoreovirus. This ground-breaking technology has led to the generation of valuable evidence regarding the replication and pathogenesis of these viruses. Unfortunately, extrapolating either the plasmid-based or transcript-based reverse genetics systems to rotavirus has not yet been successful. The development of a selection-free rotavirus reverse genetics system will enable the systematic investigation of poorly understood aspects of the rotavirus replication cycle and aid the development of more effective vaccines, amongst other research avenues.

This study investigated the importance of co-expressed rotavirus proteins in the development of a selection-free rotavirus reverse genetics system. The consensus sequences of the rotavirus strains Wa (RVA/Human-tc/USA/WaCS/1974/G1P[8]) and SA11 (RVA/Simian-tc/ZAF/SA11/1958/G3P[2]) where used to design rotavirus expression plasmids. The consensus nucleotide sequence of a human rotavirus Wa strain was determined by sequence-independent cDNA synthesis and amplification combined with next-generation 454® pyrosequencing. A total of 4 novel nucleotide changes, which also resulted in amino acid changes, were detected in genome segment 7 (NSP3), genome segment 9 (VP7) and genome segment 10 (NSP4). *In silico* analysis indicated that none of the detected nucleotide changes, and consequent amino acid variations, had any significant effect on viral structure. Evolutionary analysis indicated that the sequenced rotavirus WaCS was closely related to the ParWa and VirWa variants, which were derived from the original 1974 Wa isolate. Despite serial passaging in animals, as well as cell cultures, the Wa genome seems to be stable. Considering that the current reference sequence for the Wa strain is a composite sequence of various Wa variants, the rotavirus WaCS may be a more appropriate reference sequence.

The rotavirus Wa and SA11 strains were selected for plasmid-based expression of rotavirus proteins, under control of a T7 promoter sequence, due to the fact that they propagate well in MA104 cells and the availability of their consensus sequences. The T7 RNA polymerase was provided by a recombinant fowlpox virus. After extensive transfection optimisation on a variety of mammalian cell lines, MA104 cells proved to be the best suited for the expression rotavirus proteins from plasmids. The expression of rotavirus Wa and SA11 VP1, VP6, NSP2 and NSP5 could be confirmed with immunostaining in MA104 and HEK 293H cells. Another approach involved the codon-optimised expression of the rotavirus replication complex scaffold in MA104 cells under the control of a CMV promoter sequence. This system was independent from the recombinant fowlpox virus. All three plasmid expression sets were designed to be used in combination with the transcript-based reverse genetics system in order to improve the odds of developing a successful rotavirus reverse genetics system.

Rotavirus transcripts were generated using transcriptively active rotavirus SA11 double layered particles (DLPs). MA104 and HEK293H cells proved to be the best suited for the expression of rotavirus transcripts although expression of rotavirus VP6 could be demonstrated in all cell cultures examined (MA104, HEK 293H, BSR and COS-7) using immunostaining. In addition, the expression of transcript derived rotavirus VP1, NSP2 and NSP5 could be confirmed with immunofluorescence in MA104 and HEK 293H cells. This is the first report of rotavirus transcripts being translated in cultured cells. A peculiar cell death pattern was observed within 24 hours in response to transfection of rotavirus transcripts. This observed cell death, however does not seem to be related to normal viral cytopathic effect as no viable rotavirus could be recovered. In an effort to combine the transcript- and plasmid systems, a dual transfection strategy was followed where plasmids encoding rotavirus proteins were transfected first followed, 12 hours later, by the transfection of rotavirus SA11 transcripts. The codon- optimised plasmid system was designed as it was postulated that expression of the DLP-complex (VP1, VP2, VP3 and VP6), the rotavirus replication complex would form and assist with replication and/or packaging. Transfecting codon- optimized plasmids first noticeably delayed the mass cell death observed when transfecting rotavirus transcripts on their own. None of the examined co-expression systems were able to produce a viable rotavirus.

Finally, the innate immune responses elicited by rotavirus transcripts and plasmid-derived rotavirus Wa and SA11 proteins were investigated. Quantitative RT-PCR (qRT-PCR) experiments indicated that rotavirus transcripts induced high levels of the expression of the cytokines IFN- α1, IFN-1β, IFN-λ1 and CXCL10. The expression of certain viral proteins from plasmids (VP3, VP7 and NSP5/6) was more likely to stimulate specific interferon responses, while other viral proteins (VP1, VP2, VP4 and NSP1) seem to be able to actively suppress the expression of certain cytokines. In the light of these suppression results, specific rotavirus proteins were expressed from transfected plasmids to investigate their potential in supressing the interferon responses provoked by rotavirus transcripts. qRT-PCR results indicated that cells transfected with the plasmids encoding NSP1, NSP2 or a combination of NSP2 and NSP5 significantly reduced the expression of specific cytokines induced by rotavirus transcripts. These findings point to other possible viral innate suppression mechanisms in addition to the degradation of interferon regulatory factors by NSP1. The suppression of the strong innate immune response elicited by rotavirus transcripts might well prove to be vital in the quest to better understand the replication cycle of this virus and eventually lead to the development of a selection-free reverse genetics system for rotavirus.

Keywords:
rotavirus; transcript-based reverse genetics system; sequence-independent genome amplification; rotavirus Wa and SA11 strains; phylogenetic analysis; nucleotide substitution rate analysis; next-generation 454® pyrosequencing; consensus sequence determination; plasmid derived expression of rotavirus proteins; transfection optimisation; rotavirus transcript translation; innate immune response; interferon suppression; role of viroplasms

OPSOMMING

Tru-genetika is ’n innoverende molekulêre biologie gereedskap wat wetenskaplikes in staat stel om virale genome op cDNA vlak te manipuleer ten einde spesifieke mutante of kunsmatige virusse te genereer. Die tru-genetika sisteem vir die influensa virus is een van die beste illustrasies van die potensiële voordele van hierdie tegnologie. Hierdie tru-genetika sisteem vorm die basis vir die vermoë om die influensa vaksien variante gereeld aan te pas, om tred te hou met die nuutste seisoenale uitbrake. Tans, is daar ook tru-genetika sisteme beskikbaar vir talle RNA virusse. Seleksie-vrye tru-genetika sisteme is al ontwikkel vir lede van die *Reoviridae* familie, insluitende Afrika perde siekte virus, bloutongvirus en orthoreovirusse. Hierdie deurslaggewende tegnologie het al tot die generering van waardevolle bewyse gelei, met betrekking tot die replisering en patogenese van hierdie virusse. Ongelukkig was die ekstrapolering van die plasmied gebaseerde- sowel as die transkrip gebaseerde tru-genetika sisteme, nog onsuksesvol vir rotavirus. Die ontwikkeling van ’n seleksie-vrye tru-genetika sisteem vir rotavirus sal ’n belangrike bydrae lewer tot die sistematiese ondersoek van aspekte in die rotavirus lewensiklus waaroor daar nog onvoldoende kennis is. Sodanige begrip sal verder aanleiding gee tot die ontwikkeling van beter vaksines.

In hierdie studie word die belangrikheid van mede-uitgedrukte rotavirus proteïene in die ontwikkeling van ’n seleksie-vrye rotavirus tru-genetika sisteem ondersoek. Die konsensus volgordes van die rotavirus Wa (RVA/Human-tc/USA/WaCS/1974/G1P[8]) en SA11 (RVA/Simian-tc/ZAF/SA11/1958/G3P[2]) variante is ingespan om rotavirus uitdrukkingsplasmiede te ontwerp. Die konsensus nukleotied volgordes van die menslike rotavirus Wa variant was bepaal deur volgorde onafhanklike cDNA sintese en vermeerdering in kombinasie met volgende-generasie *454® pyrosequencing* tegnologie. ’n Totaal van 4 ongekende nukleotied veranderinge, wat ook lei tot aminosuur veranderinge, was opgemerk in genoom segment 7 (NSP3), genoom segment 9 (VP7) en genoom segment 10 (NSP4). *In silico* analises het aangedui dat geen van die opgemerkte nukleotied veranderinge, en gevolglike aminosuur veranderinge, enige merkwaardige effek op die virale struktuur het nie. Evolusionêre analises het daarop gedui dat die WaCS variant, waarvan die volgorde bepaal was, baie nou verwant is aan die ParWa en VirWa variante wat afkomstig is van die oorspronklike 1974 Wa isolaat. Ten spyte van reeks passerings in beide diere en selkulture, kom die rotavirus Wa genoom stabiel voor. As dit in ag geneem word dat die huidige verwysingsvolgorde vir rotavirus Wa saamgestel is uit verskeie Wa variante se volgordes, mag die WaCS volgorde moontlik ’n meer gepaste verwysingsvolgorde wees.

Die rotavirus Wa en SA11 variante is geselekteer vir plasmied gebaseerde uitdrukking van rotavirus proteïene, onder die beheer van ’n T7 promoter volgorde, omdat beide virusse goeie groei in MA104 selle toon, sowel as die toeganklikheid van hul konsensus volgordes. Die T7 RNA polimerase is verskaf deur ’n rekombinante voëlgriepvirus. Na ekstensiewe transfeksie optimalisering op ’n verskeidenheid soogdier sellyne, was MA104 selle die mees geskikte vir die uitdrukking van rotavirus proteïene vanuit plasmiede. Die uitdrukking van

rotavirus Wa en SA11 VP1, VP6, NSP2 en NSP5 kon bevestig word deur immunokleuring van MA104 en HEK 293H selle. ’n Alternatiewe benadering, wat die kodon geoptimaliseerde uitdrukking van die rotavirus SA11 repliseringskompleks in MA104 selle behels, is ook getoets. Hierdie stel plasmiede is onder beheer van ’n CMV promotor en is onafhanklik van die voëlgriepvirus. Al drie plasmied uitrukkingstelle was ontwerp om in kombinasie met die transkrip gebaseerde tru-genetika sisteem te werk, wat die moontlikheid vir ’n suksesvolle rotavirus tru-genetika sisteem te verbeter.

Rotavirus transkripte is voorberei deur van aktiewe rotavirus SA11 dubbellaag partikels (DLPs) gebruik te maak. MA104 en HEK 293H selle was die mees geskikte vir die uitdrukking van rotavirus transkripte, alhoewel die uitdrukking van rotavirus VP6 ook in HEK 293H, BSR en COS-7 gedemonstreer kon word deur immunokleuring. Addisioneel, kon die uitdrukking van VP1, NSP2 en NSP5 bevestig word deur immunofluorosensie in MA104 en HEK 293H selle. Hierdie is die eerste bewys van translasie van rotavirus transkripte in selkulture. ’n Opvallende seldood patroon is opgemerk binne 24 uur in reaksie op die transfeksie van rotavirus transkripte. Hierdie seldood patroon hou nie verband met normale sitopatiese effekte (CPE) nie en geen lewensvatbare virusse kon herwin word nie. In ’n poging om transkrip- en plasmiedsisteme te kombineer is ’n tweeledige transfeksie strategie gevolg waar plasmiede, wat vir rotavirus proteïene kodeer, eerste getransfekteer word, gevolg deur rotavirus transkripte, 12 ure later. Die kodon geoptimaliseerde plasmiedstel was ontwerp op grond van die hipotese dat die uitdrukking van ’n DLP-repliserings kompleks (VP1, VP2, VP3 en VP6) die replisering en verpakking van transkripte sou bystaan. As die kodon geoptimaliseerde plasmiede eerste getransfekteer is, het dit ooglopende vertraging in massa seldood, wat veroorsaak word deur transkripte, tot gevolg gehad. Geen van die mede-uitdrukkingsisteme was daartoe in staat om lewensvatbare rotavirus te lewer nie.

Laastens is die nie-spesifieke, aangebore immuunreaksie wat deur die transfeksie van rotavirus transkripte en plasmied uitgedrukte rotavirus proteïen veroorsaak word, ondersoek. Kwantitatiewe RT-PCR (qRT-PCR) eksperimente het daarop gedui dat rotavirus transkripte hoë vlakke van uitdrukking van die sitokines IFN- α1, IFN-1β, IFN-λ1 en CXCL10 induseer. Die uitdrukking van sekere rotavirus proteïene vanaf plasmiedes (VP3, VP7 en NSP5/6) het spesifieke aangebore immuunreaksies gestimuleer, terwyl ander virale proteïene (VP1, VP2, VP4 en NSP1) weer die vermoë het om sekere aangebore immuunreaksies betekenisvol te onderdruk. In die lig van hierdie onderdrukkingstendense, is die vermoë van spesifieke en/of kombinasies van rotavirus proteïene, om die aangebore immuunreaksies wat deur rotavirus transkripte uitgelok word, te onderdruk, ook ondersoek. qRT-PCR resultate het aangedui dat selle wat vooraf getransfekteer was met plamiede wat kodeer vir NSP1, NSP2 of ’n kombinasie van NSP2 en NSP5, betekenisvol die uitdrukking van spesifieke sitokines onderdruk wat deur rotavirus transkripte geïnduseer word. Hierdie bevindinge dui daarop dat daar moontlik addisionele virale meganismes is om die aangebore immuunreaksies te onderdruk, as slegs die degradering van interferon regulerende faktore deur NSP1. Die onderdrukking van die sterk aangebore immuunreaksie wat rotavirus transkripte tot gevolg het, mag uiters belangrik wees in die veldtog om die

repliseringsiklus van hierdie virus beter te verstaan en uiteindelik ’n seleksie-vrye tru-genetika sisteem vir rotavirus te ontwikkel.

Sleutel woorde:

Rotavirus; transkrip-gebaseerde tru-genetika sisteem; volgorde onafhanklike cDNA sintese en vermeerdering; rotavirus Wa en SA11 variante; filogenetiese analise; nukleotied substitusie tempo analise; 454® *pyrosequencing* tegnologie; konsensus volgorde bepaling; plasmied afkomstige uitdrukking van rotavirus proteïene; transfeksie optimalisering; rotavirus transkrip translasie; aangebore immuunreaksie; interferon onderdrukking; rol van viroplasmas

PUBLICATIONS ASSOCIATED WITH THIS STUDY

Published 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

Submitted manuscript:

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CONFERENCE CONTRIBUTIONS

Poster presentations:

Wentzel, J.F., van Dijk, A.A. and O'Neill H.G. 2011. DETERMINATION OF THE ROTAVIRUS Wa CONSENSUS SEQUENCE BY SEQUENCE INDEPENDENT cDNA SYNTHESIS AND AMPLIFICATION COMBINED WITH 454 PYROSEQUENCING. Virology Africa Congress. Cape Town, South Africa

Wentzel, J.F., Yuan, L., Roa, 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 DERIVED FROM THE ORIGINAL Wa ISOLATE. 5th European Rotavirus Biology Meeting, Valencia, Spain

Wentzel, J.F., du Plessis L. H., Mlera, L. van Dijk, A.A. and O'Neill H.G. 2013.SUPPRESSING THE INTERFERON RESPONSE ELICITED BY ROTAVIRUS TRANSCRIPTS USING PLASMID DERIVED ROTAVIRUS NON-STRUCTURAL PROTEINS NSP1, NSP2 and NSP5/6. 5th European Rotavirus Biology Meeting, Valencia, Spain