Preparatory investigations for developing a transcript-based rotavirus reverse genetics system

By

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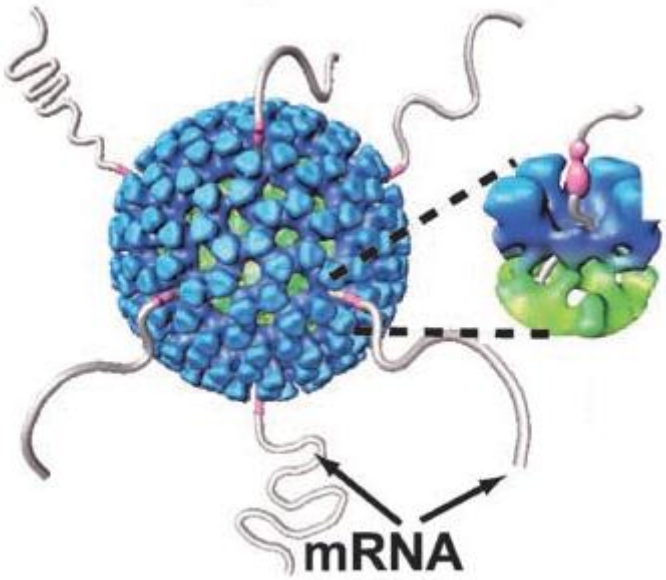

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The cover image depicts a double-layered rotavirus particle with transcripts exiting through type I channels at five-fold vertices. Figure from Prasad *et al*., 2006 with permission from the publisher.

Wisdom is the right use of knowledge. To know is not to be wise. Many men know a great deal, and are all the greater fools for it. There is no fool so great a fool as a knowing fool. But to know how to use knowledge is to have wisdom.

Charles Spurgeon

Everybody is a genius. But if you judge a fish by its ability to climb a tree, it will live its whole life believing that it is stupid."

Albert Einstein

To my daughters Laura Tadiwa and Adriel Maka who thought that when I was not at home I was in the lab, everywhere!

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Abbreviations

AHSV: African horse-sickness virus

ATP: adenosine triphosphate

bp: base pairs

BTV: bluetongue virus

CD4: cluster of differentiation 4

CD8: cluster of differentiation 8

CPE: cytopathic effect

CsCl: cesium chloride

CTP: cytosine triphosphate

DNA: deoxyribonucleic acid

DLP: double-layered particle

dsRNA: double-stranded ribonucleic acid

EB: elution buffer

EDTA: ethylene-diamine-tetra-acetic acid

ELISA: enzyme-linked immunosorbent assays

ER: endoplasmic reticulum

FBS: foetal bovine serum

ffu: focus forming units

GTP: guanosine triphosphate

HA: haemagglutinin

HEK: human embryonic kidney

HIV: human immunodeficiency virus

IFN: interferon

IgA: Immunoglobulin A

IgG: Immunoglobulin G

IgY: immunoglobulin Y

kDa: kiloDalton

LPG-2: laboratory of physiology and genetics gene 2

MCRI: Murdoch Children’s Research Institute

MDA5: melanoma differentiation associated gene 5

MOI: multiplicity of infection

ml: millilitre

NA: neuraminidase

NTPase: nucleoside triphosphatase

NSP: non-structural protein

ORF: open reading frame

PABP: poly (A) binding proteins

PAGE: polyacrylamide gel electrophoresis

PBS: phosphate-buffered saline

pDC: plasmacytoid dendritic cell

PCR: polymerase chain reaction

pfu: plaque forming units

PKR: dsRNA-dependent kinase

RdRp: RNA-dependent RNA polymerase

RIG-I: retinoic inducible gene I

RNA: ribonucleic acid

RNAi: ribonucleic acid interference

rpm: revolutions per minute

qRT-PCR: quantitative reverse transcription polymerase chain reaction

siRNA: small interfering ribonucleic acid

SA11: simian agent 11

SAGE: Strategic Advisory Group of Experts

ssRNA: single-stranded ribonucleic acid

SDS-PAGE: sodium dodecyl sulphate polyacrylamide gel electrophoresis

siRNA: small interfering RNA

TLP: triple-layered particle

TBST: Tris buffered saline with Tween-20

U: unit

USA: united States of America

UTR: untranslated terminal region

VP: structural viral protein

^{o}C: degrees Celsius

Summary

Reverse genetics systems that are based on either viral transcripts or cDNA genome segments cloned in plasmids have recently been reported for some of the dsRNA viruses of the *Reoviridae* family, namely African horsesickness virus, bluetongue virus and orthoreovirus. For rotaviruses, three reverse genetics systems which only allow the manipulation of a single genome segment have been described. These rotavirus single genome segment reverse genetics systems are not true stand-alone systems because they require a helper virus and a recombinant virus selection step. A true selection-free, plasmid- only or transcript-based reverse genetics system for rotaviruses is lacking.

This study sought to identify and characterise the factors that need to be understood and overcome for the development of a rotavirus reverse genetics system using mRNA derived from the *in vitro* transcription of a consensus nucleotide sequence as well as from double-layered particles. The consensus whole genome sequence of the prototype rotavirus DS-1 and SA11 strains was determined using sequence-independent whole genome amplification and 454® pyrosequencing. For the rotavirus DS-1 strain, a novel isoleucine in a minor population variant was found at position 397 in a hydrophobic region of VP4. NSP1 contained seven additional amino acids MKSLVEA at the N-terminal end due to an insertion in the consensus nucleotide sequence of genome segment 5. The first 34 nucleotides at the 5'-terminus and last 30 nucleotides at the 3'-terminal end of genome segment 10 (NSP4) of the DS-1 strain were determined in this study. The consensus genome segment 11 (NSP5/6) sequence was 821 bp in length, 148 bp longer than previously reported. The 454® pyrosequence data for a rotavirus SA11 sample with no known passage history revealed a mixed infection with two SA11 strains. One of the strains was a reassortant which contained genome segment 8 (NSP2) from the bovine rotavirus O agent. The other ten consensus genome segments of the two strains could not be differentiated. Novel minor population variants of genome segments 4 (VP4), 9 (VP7) and 10 (NSP4) were identified. Molecular clock phylogenetic analyses of the rotavirus SA11 genomes showed that the two SA11 strains were closely related to the original SA11-H96 strain isolated in 1958.

Plasmids containing inserts of the consensus cDNA of the rotavirus DS-1 strain were purchased and used to generate exact capped transcripts by *in vitro* transcription with a T7 polymerase. Wild-type transcripts of rotavirus SA11 were obtained from *in vitro* transcription using purified rotavirus SA11 double-layered particles. The purified rotavirus DS-1 and SA11 transcripts were transfected into BSR, COS-7 and MA104 cells. Work on MA104 cells was discontinued due their very low transfection efficacy. In BSR and COS-7 cells, rotavirus DS-1 and SA11 transcripts induced cell death. However, no viable rotavirus was recovered following attempts to infect MA104 cells with the BSR and COS-7 transfected cell lysates. The cell death was determined to be due to apoptotic cell death mechanisms. Immunostaining showed that the DS-1 genome segment 6 (VP6) and SA11 transcripts were translated in transfected BSR and COS-7 cells. Based on visual inspection, the translation seemed to be higher in the retinoic acid-inducible gene-I (RIG-I) deficient BSR cells than in COS-7 cells. This suggested that the transfection of rotavirus transcripts induced an innate immune response which could lead to the development of an antiviral state. Therefore, the innate immune response to rotavirus transcripts was investigated in HEK 293H cells using qRT-PCR and western blot analyses. Results of this investigation showed that RIG-I, but not MDA5 sensed rotavirus transcripts in transfected HEK 293H cells. Furthermore, rotavirus transcripts induced high levels of cellular mRNA encoding the cytokines IFN-1β, IFN-λ1, CXCL10 and TNF-α. Other cytokines namely, IFN-α, IL-10, IL-12 p40 and the kinase RIP1 were not significantly induced. Inhibiting the RNA-dependent protein kinase R (PKR) reduced the induction of cytokines IFN-1β, IFN-λ1, CXCL10 and TNF-α, but the expression levels were not abrogated. The importance of a consensus sequence and the insights gained in the current study regarding the role of the innate immune response after transfection of rotavirus transcripts into cells in culture, should aid the development of a true rotavirus reverse genetics system.

Key words

Rotavirus; rotavirus DS-1 strain; rotavirus SA11 strain; dsRNA; sequence-independent genome amplification; 454® pyrosequencing; consensus genome sequence; *in vitro* transcription; reverse genetics; transfection; innate immune response; antiviral state.

Opsomming

Tru-genetika sisteme wat gebaseer is op transkripsie produkte van virusse of kDNS genoom segmente wat in plasmiede gekloneer is, is onlangs gerapporteer vir sommige ddRNS virusse van die *Reoviridae* familie, naamlik Afrika perdesiekte virus, bloutongvirus en ortoreovirus. Vir rotavirusse is drie tru-genetika sisteme beskryf wat toelaat dat net ‘n enkele genoom segment gemanipuleer kan word. Hierdie rotavirus enkel genoom segment tru-genetika sisteme is nie ware alleenstaande sisteme nie, want hulle benodig ‘n helper virus en ‘n stap om rekombinante virus te selekteer. Daar is nog nie ‘n ware seleksie-vrye, slegs plasmied of transkrip gebaseerde tru-genetika sisteem vir rotavirusse nie.

Met hierdie studie is gepoog om die faktore te identifiseer en te karakteriseer wat verstaan en oorkom moet word in die ontwikkeling van ‘n rotavirus tru-genetika sisteem wat mRNA gebruik wat afkomstig is van *in vitro* transkripsie van ‘n konsensus nukleotied volgorde sowel as van dubbellaag partikels. Die konsensus heel-genoom volgorde van die prototipe rotavirus DS-1 en SA11 stamme is bepaal deur volgorde-onafhanklike heel genoom vermeerdering en 454® pirovolgorde bepaling te gebruik. In die rotavirus DS-1 stam is ‘n nuwe isoleusien gevind in ‘n minderheids populasie variant in posisie 397 in ‘n hidrofobe gebied van VP4. NSP1 het sewe addisionele aminosure MKSLVEA in die N-terminale ent weens ‘n invoeging in die konsensus nukleotiedvolgorde van genoom segment 5. Die eerste 34 nukleotiede van die 5’-terminus en die laaste 30 nukleotiede van die 3’-terminale ent van genoom segment 10 (NSP4) van die DS-1 stam is in hierdie studie bepaal. Die konsensus genoom segment 11(NSP5/6) volgorde was 821 bp lank, 148 bp langer as wat vantevore gerapporteer is. Die 454® pirovolgorde bepalings data van ‘n SA11 rotavirus monster sonder enige passasie geskiedenis het ‘n gemenge infeksie met twee SA11 stamme onthul. Een van die stamme was ‘n hergegroepeerde virus wat genoom segment 8 (NSP2) van die bees rotavirus O agent bevat het. Die ander tien konsensus genoom segmente kon nie onderskei word nie. Nuwe minderheids populasie variante van genoom segmente 4 (VP4), 9 (VP7) en 10 (NSP4) is geïdentifiseer. Molekulêre klok filogenetiese analises van die SA11 genoom het getoon dat die twee SA11 stamme naby verwant is aan die oorspronklike SA11-H96 stam wat in 1958 geïsoleer is.

Plasmiede wat insetsels van die konsensus kDNS van die rotavirus DS-1 stam bevat, is aangekoop en gebruik om presiese bedekte (“capped”) transkripte te genereer deur *in vitro* transkripsie met ‘n T-7 polimerase. Wilde-tipe transkripte van rotavirus SA11 is verkry van *in vitro* transkripsie deur gebruik te maak van gesuiwerde SA11 dubbellaag partikels. Die gesuiwerde rotavirus DS-1 en SA11 transkripte is getransfekteer in BSR, COS-7 en MA104 selle. Werk met die MA104 selle is gestaak weens hulle baie lae transfeksie effektiwiteit. In BSR en COS-7 selle het DS-1 en SA11 transkripte seldood geïnduseer. Daar is egter geen lewensvatbare rotavirus herwin na pogings om MA104 selle te infekteer met die BSR en COS-7 getransfekteerde sel-lisate nie. Daar is vasgestel dat die seldood toe te skryf was aan apoptotiese seldood meganismes. Immunokleuring het gewys dat die DS-1 genoom segment 6 (VP6) en SA11 transkripte getransleer is in getransfekteerde BSR en COS-7 selle. Gebasseer op visuele inspeksie, het dit gelyk asof die translasie in die retinoësuur-induseerbare geen-1 (RIG-1) defekte BSR selle hoër was as in COS-7 selle. Dit was aanduidend daarvan dat die transfeksie van rotavirus transkripte ‘n ingebore immuunrespons geïnduseer het wat kan lei tot die ontwikkeling van ‘n teenvirus staat. Daarom is die ingebore immuunrespons op rotavirus transkripte bestudeer in HEK 293H selle deur qRT-PKR en westelike klad analises. Resultate van hierdie ondersoek het gewys dat rotavirus transkripte deur RIG-1, maar nie MDA5 waargeneem word in getransfekteerde HEK 293H selle. Daarbenewens toon die resultate aan dat die rotavirus transkripte hoë vlakke van sellulêre mRNA geïnduseer het wat kodeer vir die sitokine IFN-1β, IFN-λ1, CXCL10 en TNF-α. Ander sitokine, naamlik IFN-α, IL-10, IL-12 p40 en die kinase RIP1 is nie betekenisvol geïnduseer nie. Onderdrukking van die RNS-afhanklike proteïen kinase R (PKR) het die induksie van die sitokine IFN-1β, IFN-λ1, CXCL10 en TNF-α verlaag, alhoewel die vlakke van uitdrukking nie heeltemal onderdruk was nie. Die belang van die konsensus genoom opeenvolging en die insae verkry in die huidige studie betreffende die rol van die ingebore immuunrespons na transfeksie van rotavirus transkripte in selkulture behoort te help met die ontwikkeling van ’n ware rotavirus tru-genetika sisteem.

Sleutelwoorde

Rotavirus; rotavirus DS-1 stam; rotavirus SA11 stam; ddRNS; volgorde-onafhanklike genoom vermeerdering; 454® pirovolgordebepaling; konsensus genoom volgorde; *in*

vitro transkripsie; tru-genetika; transfeksie; ingebore immuunrespons; teenvirus toestand

Publications associated with this study

- Jere, K.C., **Mlera, L**., O'Neill, H.G., and van Dijk A.A (2012). Whole genome sequence analyses of three African bovine rotaviruses reveal that they emerged through multiple reassortment events between rotaviruses from different mammalian species. *Veterinary Microbiology,* 159: 245–250

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- **Luwanika Mlera**, Hester G. O'Neill, Khuzwayo C. Jere, and Alberdina A. van Dijk (2011). Evolution of the consensus whole-genome of a South African rotavirus SA11 obtained with 454® pyrosequencing: evidence of a mixed infection with two close derivatives of the SA11-H96 strain. *Archives of Virology*, Accepted 18 October 2012, Available online 23 December 2012.

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- **L. Mlera**, A. A. van Dijk and H. G. O'Neill. Consensus genome sequence of SA11 rotavirus determined with sequence-independent genome amplification and 454® pyrosequencing. **Virology Africa 2011 Conference**, Cape Town Waterfront, 29 November 2011–2 December 2011 [Poster].

- Jere, K.C**., Mlera, L**., Page, N.A., van Dijk A.A., and O'Neill, H.G**.** Evidence that mixed infections promotes generation of novel strains through intragenogroup and intergenogroup genome recombination revealed through whole genome characterization of multiple rotavirus strains from a single stool specimen. **Vaccine for Enteric Diseases,** 13th -16th September 2011, The Novotel Cannes Montfleury, Cannes, France [Poster].

- Jere, K.C., (presenter) **Mlera, L**., Page, N.A., van Dijk A.A., & O'Neill, H.G**.** Evidence that mixed infections promotes generation of novel strains through intragenogroup and intergenogroup genome recombination revealed through whole genome characterization of multiple rotavirus strains from a single stool specimen. **The Malawi-Liverpool Wellcome Trust Research 2011 Conference**, 18th -24th September, 2011, Club Makokola, Mangochi, Malawi [Oral].

- **L. Mlera**, K.C. Jere, A.C. Potgieter, A.A. van Dijk and H.G. O'Neill. Molecular characterization of the DS-1 rotavirus strain using 454® pyrosequencing technology. **9th International Rotavirus Symposium**, Johannesburg, South Africa, 2-3 August 2010 [Poster].

- **L. Mlera**, K.C. Jere, A.C. Potgieter, A.A. van Dijk and H.G. O'Neill. Molecular characterization of the DS-1 rotavirus strain using 454® pyrosequencing technology. **6th African Rotavirus Symposium**, Johannesburg, South Africa, 4 August 2010 [Poster].

- Jere, K.C., **Mlera, L**., O'Neill, H.G., Potgieter, A.C., Page, N.A., Peenze, I., & van Dijk A.A. Sequence-independent amplification and ultra-deep sequencing of the emerging and prevalent African rotavirus strains. **6th African rotavirus symposium,** 4th August 2010, National Institute for Communicable Diseases, Johannesburg, South Africa [Poster].

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