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Whole genome characterisation and engineering of chimaeric rotavirus-like particles using African rotavirus field strains

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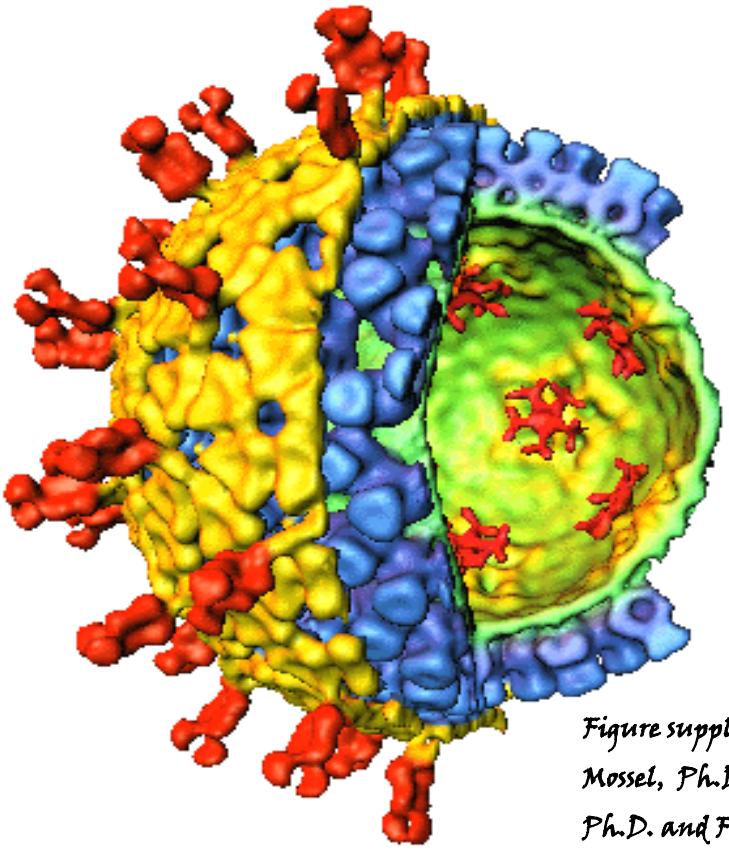

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Co-Promoter: Dr H. G. (Trudi) O'Neill
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"Every great dream begins with a dreamer. Always remember, you have within you the strength, the patience, and the passion to reach for the stars to change the world."
Harriet Tubman

"Go confidently in the direction of your dreams. Live the life you have imagined."
Henry David Thoreau

"Reach high, for stars lie hidden in your soul. Dream deep, for every dream precedes the goal."
Pamela Vaull Starr

"All men dream but not equally. Those who dream by night in the dusty recesses of their minds wake in the day to find that it was vanity; but the dreamers of the day are dangerous men, for they may act their dream with open eyes to make it possible."
T.E. Lawrence

"Our truest life is when we are in dreams awake"
Henry David Thoreau

So often times it happens that we live our lives in chains
And we never even know we have the key.

From Already Gone, performed by the Eagles for their 1974 On the Border album.

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ABBREVIATIONS

aa:	Amino acid
ACIP :	Advisory Committee on Immunization Practice
AcMNPV-Sf9:	*Autographa californica* multi-capsid nucleopolyhedrosis virus-*Spodoptera frugiperda* 9
AGMK:	African green monkey kidney
ATP:	Adenosine triphosphate
BCA:	Bicinchoninic acid
bp:	Base pairs
BVES:	Baculovirus vector expression system
CAI:	Codon adaptation index
cfu:	Colony forming units
CPE:	Cytopathic effect
CsCl:	Cesium chloride
cRV-VLP:	Complete rotavirus virus-like particles
Da:	Dalton
$_{dd}H_2O$	Double-distilled water
DNA:	Deoxyribonucleic acid
DLP:	Double-layered particle
DPI:	Days post infection
DRC:	Democratic Republic of Congo
dRV-VLP:	Double-layered rotavirus virus-like particle
dsRNA:	Double-stranded ribonucleic acid
EIA:	Enzyme immune assays
EB:	Elution buffer
EC:	Enterochromaffin cells
EDIM:	Epizootic diarrhoea of infant disease
EDTA:	Ethylene-diamine-tetra-acetic acid
ELISA:	Enzyme-linked immunosorbent assays
EM:	Electron microscope
ENS:	Enteric nervous system
ER:	Endoplasmic reticulum
EPI:	Expanded programme on immunization

FBS:	Foetal bovine serum
FDA:	Food and Drug Administration
GSK:	GlaxoSmithKline
HA:	Haemagglutinin
HIV:	Human immunodeficiency virus
HPI:	Hours post infection
H:	Hour
HSC70:	Heat-shock cognate 70 proteins
ICTV:	International Committee on Taxonomy of Viruses
IgA:	Immunoglobulin A
IgG:	Immunoglobulin B
IgM:	Immunoglobulin M
IPTG:	Isopropyl β-D-1-thiogalactopyranoside
LB:	Lysogeny broth
kDa:	KiloDalton
MDG:	Millennium Development Goals
MID:	Multiplex identifier
MOI:	Multiplicity of infection
MTA:	Material transfer agreements
UL, MEDUNSA:	University of Limpopo, Medical University of Southern Africa Campus
ml:	Millilitre
NA:	Neuraminidase
NICD:	National Institute for Communicable Diseases of South Africa
NIH:	National Institutes of Health
nt:	Nucleotide
NTPase:	Nucleosidetriphosphatase
NWU:	North-West University
NSP:	Non-structural protein
OD:	Optical density
ORF:	Open reading frame
ORS:	Oral rehydration solution
OVI:	Onderstepoort Veterinary Institute
PABP:	Poly (A) binding proteins
PAGE:	Polyacrylamide gel electrophoresis

PBS:	Phosphate-buffered saline
PCR:	Polymerase chain reaction
PCV1:	Porcine circovirus type 1
pfu:	Plaque forming units
PLC:	Phospholipase C
QWBZP:	Qiwei Baizhu Powder
RCWG:	Rotavirus Classification Working Group
RdRp:	RNA-dependent RNA polymerase
RE:	Restriction endonuclease enzyme
RNA:	Ribonucleic acid
RPM:	Revolution per minute
RT-PCR:	Reverse Transcription polymerase chain reaction
RV-VLP:	Rotavirus virus-like particle
SAP:	Shrink alkaline phosphotise
SARS:	Severe Acute Respiratory Syndrome
siRNA:	Small interfering ribonucleic acid
sRV-VLP:	single-layered rotavirus virus-like particle
SOC:	Super Optimal broth with catabolite repression
ssRNA:	Single-stranded ribonucleic acid
SDS-PAGE:	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Sf9:	*Spodoptera frugiperda 9*
SA:	Sialic acid
Sec:	Seconds
siRNA:	Small interfering RNA
SLP:	Single-layered particle
SOC:	Super optimal broth with catabolite repression
TEEDTA:	Tris-acetate-ethylene-diamine-tetra-acetic acid
TEM:	Transmission electron microscopy
TGS:	Tris-glycine-sodium dodecyl sulphate
TLP:	Triple-layered particle
TOI:	Time of infection
TNT buffer:	0.05% Tween, 0.2 M NaCl and 0.05 M Tris-HCl buffer
tRV-VLP:	Triple-layered rotavirus virus-like particle
U:	Unit
UCT:	University of Cape Town

UNICEF:	United Nations Children’s Fund
USA:	United States of America
UTR:	Untranslated terminal region
VLP:	Virus-like particles
V:	Volts
VP:	Structural viral protein
WHO:	World Health Organisation
5-HT:	5-hydroxytryptamine
^{o}C:	Degrees Celsius

SUMMARY

Despite the global licensure of two live-attenuated rotavirus vaccines, Rotarix® and RotaTeq®, rotavirus remains the major cause of severe dehydrating diarrhoea in young mammals and the need for further development of additional rotavirus vaccines, especially vaccines effective against regional strains in developing country settings, is increasing. The design and formulation of new effective multivalent rotavirus vaccines is complicated by the wide rotavirus strain diversity. Novel rotavirus strains emerge periodically due to the propensity of rotaviruses to evolve using mechanisms such as point mutation, genome segment reassortment, genome segment recombination and interspecies transmission. Mutations occurring within the primer binding regions targeted by the current commonly employed sequence-dependent genotyping techniques lead to difficulties in genotyping novel mutant rotavirus strains. Therefore, use of sequence-independent techniques coupled with online rotavirus genotyping tools will help to understand the complete epidemiology of the circulating strains which, in turn, is vital for developing intervention measures such as vaccine and anti-viral therapies.

In this study, sequence-independent cDNA synthesis that uses a single set of oligonucleotides that do not require prior sequence knowledge of the rotavirus strains, 454® pyrosequencing, and an online rotavirus genotyping tool, RotaC, were used to swiftly characterise the whole genome of rotaviruses. The robustness of this approach was demonstrated in characterising the complete genetic constellations and evolutionary origin of selected human rotavirus strains that emerged in the past two decades worldwide, human rotavirus strains frequently detected in Africa, and the whole genomes of some common strains frequently detected in bovine species. Most of the characterised strains emerged either through intra- or inter-species genome segment reassortment processes. The methods used in this study also allowed determination of the whole consensus genome sequence of multiple rotavirus variants present in a single stool sample and the elucidation of the evolutionary mechanisms that explained their origin. The 454® pyrosequence-generated data revealed evidence of intergenotype rotavirus genome segment recombination between the genome segments 6 (VP6), 8 (NSP2) and 10 (NSP4) of Wa-like and DS-1-like origin.

The use of next generation sequencing technology combined with sequence-independent amplification of the rotavirus genomes allowed the determination of the consensus nucleotide sequence for each of the genome segments of the selected study strains directly from stool sample.

The consensus nucleotide sequences of the genome segments encoding VP2, VP4, VP6 and VP7 of some of the study strains were codon optimised for insect cell expression and used to generate recombinant baculoviruses. The Bac-to-Bac baculovirus expression system was used to generate chimaeric rotavirus virus-like particles (RV-VLPs). These chimaeric RV-VLPs contained inner capsids (VP2 and VP6) derived from a South African RVA/Human-wt/ZAF/GR10924/1999/G9P[6] strain, on to which outer capsid layer proteins composed of various combinations of VP4 and VP7 were assembled. The outer capsid proteins were derived from the dsRNA of G2, G8, G9 or G12 strains associated with either P[4], P[6] or P[8] genotypes that were directly extracted from human stool faecal specimens. The structures of these chimaeric RV-VLPs were morphologically evaluated using transmission electron microscopy (TEM). Based on the size and morphology of the particles, double-layered (dRV-VLPs) and triple-layered RV-VLPs (tRV-VLPs) were produced. Recombinant rotavirus proteins readily assembled into dRV-VLPs, whereas approximately 10 – 30% of the assembled RV-VLPs from insect expressed recombinant VP2/6/7/4 were chimaeric tRV-VLPs. These RV-VLPs will be evaluated in future animal studies as potential non-live rotavirus vaccine candidates. The novel approach of producing RV-VLPs introduced in this study, namely by using the consensus nucleotide sequence derived from dsRNA extracted directly from clinical specimens, should speed up vaccine research and development by bypassing the need to adapt the viruses to tissue culture and circumventing some other problems associated with cell culture adaptation as well. Thus, it is now possible to generate RV-VLPs for evaluation as non-live vaccine candidates for any human or animal field rotavirus strain.

Keywords:

Human rotavirus; bovine rotavirus; 454® pyrosequencing; sequence-independent genome amplification; whole genome analysis; genome segment reassortment; genome segment recombination; mixed infection; emerging rotavirus strains; rotavirus genogroup; rotavirus virus-like particles.

OPSOMMING

Ondanks die wêreldwye lisensiëring van twee lewend ge-attenueerde rotavirus entstowwe, Rotarix® en RotaTeq®, bly rotavirus die hoofoorsaak van ernstige ontwaterende diarree in jong soogdiere en neem die noodsaaklikheid vir die ontwikkeling van addisionele rotavirus entstowwe, veral entstowwe wat effektief teen plaaslike stamme in ontwikkelende lande is, toe. Die ontwikkeling en formulering van nuwe effektiewe multivalente rotavirus entstowwe word gekompliseer deur die wye verskeidenheid rotavirus stamme wat bestaan. Nuwe rotavirus stamme ontstaan periodiek weens die vermoë van rotavirusse om te verander deur meganismes soos mutasie, genoomsegment-uitruiling, genoomsegment-rekombinasie en interspesie oordrag. Mutasies wat voorkom in die voorvoerder-bindingsgebiede wat geteiken word deur die huidige, algemeen gebruikte, volgorde-afhanklike genotiperingstegnieke, lei tot probleme met genotipering van nuwe mutante rotavirus stamme. Daarom mag die gebruik van volgorde-onafhanklike metodes gekoppel met aanlyn rotavirus genotiperings hulpmiddels help met die opklaring van die volledige epidemiologie van sirkulerende stamme, wat op sy beurt weer krities is vir die ontwikkeling van voorkomings- en behandelingsmetodes soos entstowwe en anti-virus terapieë.

In hierdie studie is volgorde-onafhanklike komplementêre DNA sintese wat 'n enkele stel oligonukleotied-voorvoerders gebruik waarvoor geen bestaande kennis van volgordes nodig is nie, 454® pirobasevolgordebepaling en 'n aanlyn rotavirus genotiperingshulpmiddel, RotaC, gebruik om die volledige volgorde van die genome van rotavirusse vinnig te karakteriseer. Die kragtigheid van hierdie metode is gedemonstreer deur die karakterisering van die volledige genetiese konstellasie en evolusionêre oorsprong van die gekose menslike rotavirusstamme wat in die afgelope twee dekades te voorskyn gekom het, mens rotavirusstamme wat dikwels in Afrika waargeneem word en die volledige genome van 'n paar stamme wat algemeen in beeste voorkom. Meeste van die gekarakteriseerde studiestamme het ontstaan deur òf intra- òf interspesie genoomsegment uitruilingsprosesse. Die metodes wat in hierdie studie gebruik is, het dit ook moontlik gemaak om die hele genoom se konsensus basevolgorde van verskeie rotavirusstamme wat in 'n enkele stoelgang monster teenwoordig was tydens 'n gemengde infeksie te bepaal, sowel as die evolusionêre meganismes wat hulle oorsprong verklaar. Die data wat met pirobasevolgordebepaling gegenereer is, het bewys gelewer van intergenotipe genoomsegment rekombinasie tussen

genoom segmente 6 (VP6), 8 (NSP2) en 10 (NSP4) van Wa-agtige en DS-1-agtige oorsprong.

Die gebruik van massiewe parallelle volgende-generasie basevolgordebepalingstegnologie gekombineer met volgorde-onafhanklike vermeerdering van die rotavirus genome, het dit moontlik gemaak om die konsensus nukleotiedvolgordes van elkeen van die genoomsegmente van die gekose studiestamme direk van stoelgang monsters te bepaal. Die konsensus nukleotiedvolgordes van die genoomsegmente wat kodeer vir VP2, VP4, VP6 en VP7 van sommige van die studiestamme se kodons is ge-optimiseer vir insekseluitdrukking en gebruik om chimeriese rotavirus virusagtige partikels (RV-VAPs) te berei. Hierdie chimeriese RV-VAPs het die binnedop (VP2 en VP6) van 'n Suid-Afrikaanse RVA/Mens-wt/ZAF/GR10924/1999/G9P[6] stam bevat waarop die buitedop proteïene bestaande uit verskillende kombinasies van VP4 en VP7 geheg is. Die buitedop proteïene is verkry vanaf dubbeldraad RNA van G2, G8, G9 of G12 stamme in assosiasie met òf P[4], P[6] òf P[8] genotipes wat direk uit menslike stoelgang monsters gehaal is. Die strukture van hierdie chimeriese RV-VAPs is morfologies geëvalueer met behulp van transmissie elektronmikroskopie (TEM). Gebaseer op die grootte en morfologie van die partikels is vasgestel dat dubbellaag (dRV-VAPs) en trippellaag (tRV-VAPs) partikels geproduseer is. Rekombinante rotavirus proteïene het geredelik saamgegroepeer om dRV-VAPs te vorm, maar net 10-30% van die saamgegroepeerde RV-VAPs van die insekseluitgedrukte rekombinante VP2/6/7/4 was tRV-VAPs. Hierdie RV-VAPs sal in toekomstige diere studies as potensiële nie-lewendige rotavirus entstof kandidate geëvalueer word. Die nuwe benadering om RV-VAPs te berei, gedemonstreer in hierdie studie, naamlik om die konsensus nukleotiedvolgorde te gebruik wat verkry is vanaf dubbeldraad RNA wat direk vanuit kliniese monsters gehaal is, behoort navorsing en ontwikkeling van entstowwe te bespoedig deur die noodsaaklikheid vir selkultuur aanpassing te oorkom asook ander probleme wat met selkultuur aanpassing geassosieer is. Derhalwe is dit nou moontlik om RV-VAPs vir evaluering as nie-lewendige enstofkandidate voor te berei vir enige menslike of diere veld rotavirusstam.

Sleutelwoorde:

Menslike rotavirus; bees rotavirus; 454® pirobasevolgordebepaling; volgorde-onafhanklike genoom vermeerdering; volle genoom analiese; genoomsegment uitruiling; genoomsegment rekombinasie; gemengde infeksie; ontluikende rotavirus stamme; rotavirus genogroep; rotavirus virusagtige partikels.

LIST OF PUBLICATIONS RELATED TO THIS STUDY

(FEBRUARY 2009 – APRIL 2012)

- **Jere, K.C.,** Mlera, L., O'Neill, H.G., & van Dijk A.A. *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,** Available online, 6 April 2012. http://dx.doi.org/10.1016/j.vetmic.2012.03.040

- **Jere, K.C.,** Mlera, L., Page, N.A., van Dijk A.A., & O'Neill, H.G. *Whole genome analysis of multiple rotavirus strains from a single stool specimen using sequence-independent amplification and 454® pyrosequencing reveals evidence of intergenotype genome segment recombination.* **Infection, Evolution and Genetics**, Dec 2011, Vol 11(8):p2072-2082.

- **Jere, K.C.,** Mlera, L., O'Neill, H.G., Potgieter, A.C., Page, N.A., Seheri, M.L., & van Dijk A.A. *Whole genome analyses of African G2, G8, G9 and G12 rotavirus strains using sequence-independent amplification and 454® pyrosequencing.* **Journal of Medical Virology**, Nov 2011, Vol 83: p2018-2042.

- Mlera, L., **Jere, K. C.,** van Dijk, A. A., & H. G. O'Neill. *Determination of the whole-genome consensus sequence of the prototype DS-1 rotavirus using sequence-independent genome amplification and 454® pyrosequencing.* **Journal of Virological Methods**; May 2011, Vol 175: 266–271.

- Nyaga, M.M., **Jere, K.C.,** Peenze, I., Mlera, L., Van Dijk, A.A., Seheri, M.L., Mphahlele, M.J.. *Sequence analysis of the complete genomes of five African human G9 rotavirus strains.* Manuscript in preparation (To be submitted to **Archives of Virology**, May 2012).

- Mlera, L., **Jere, K. C.,** van Dijk, A. A., & H. G. O'Neill. *Whole-genome consensus sequence of the model SA11 rotavirus determined with sequence-independent genome amplification and 454® pyrosequencing.* Manuscript in preparation (Submitted to **Infection, Genetics and Evolution** journal (April 2012).

CONFERENCE AND WORKSHOP PRESENTATION DURING THE STUDY PERIOD

(FEBRUARY 2009 – APRIL 2012)

- O'Neill, H.G., (presenter) Van der Westhuizen M.J., **Jere, K.C.,** Potgieter, A.C., & van Dijk A.A. *Production of rotavirus- like particles in insect cells using the codon optimised consensus sequence of a South African G9P[6] strain.* **4th European rotavirus biology meeting,** 2nd -5th October, 2011, Altafiumara – Santa Trada di Cannitello, Villa San Giovanni (RC), *Italy.*

- **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.* **Vaccine for Enteric Diseases,** 13th - 16th September 2011, The Novotel Cannes Montfleury, Cannes, *France.*

- **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.*

- **Jere, K.C.,** (presenter) 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.* **6thAfrican rotavirus symposium,** 4th August 2010, National Institute for Communicable Diseases, Johannesburg, *South Africa.*

- **Jere, K.C.,** (presenter) 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.* **9th International rotavirus symposium,** 2-3rd August 2010, Johannesburg, *South Africa.*

- L. Mlera., (presenter) **Jere, K. C.,** Potgieter, A.C., van Dijk, A. A., & H. G. O'Neill**.** Molecular characterization of the DS-1 rotavirus strain using *454® pyrosequencing.* **6th African rotavirus symposium,** 4th August 2010, National Institute for Communicable Diseases, Johannesburg, *South Africa.*

- L. Mlera., (presenter) **Jere, K. C.,** Potgieter, A.C., van Dijk, A. A., & H. G. O'Neill**.** *Molecular characterization of the DS-1rotavirus strain using 454® pyrosequencing.* **9th International rotavirus symposium,** 2-3rd August 2010, Johannesburg, *South Africa.*

- **Jere, K.C.,** (presenter) O'Neill, H. G., & van Dijk, A. A. *Strategies to construct a potential cost effective and safe chimaeric virus-like particle that will be utilized as a subunit rotavirus vaccine*. **15th International bioinformatics workshop on virus evolution and molecular epidemiology,** 7-11th September 2009, Rotterdam, *Netherlands.*