Rapid reverse transcriptase recombinase polymerase amplification assay for flaviviruses using non-infectious in vitro transcribed RNA as positive controls

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Highlights

- West Nile and Wesselsbron viruses cause outbreaks of disease in southern Africa.
- Isothermal assays have been proposed as fieldable nucleic acid amplification tests.
- Non infections transcribed RNA was used to optimise and validate an isothermal RT-RPA.
- Amplification products were detected using lateral flow devices.
- Specific probe design allowed differentiation of West Nile and Wesslesbron viruses.

Abstract

West Nile virus (WNV) and Wesselsbron virus (WSLV) are mosquito-borne viruses belonging to the *Flavivirus* genus, family *Flaviviridae* and cause outbreaks in southern Africa after heavy rain. Isothermal assays have been proposed for application in field situations as well as low resource settings and hence we developed a reverse-transcriptase recombinase polymerase amplification (RT-RPA) to detect WNV and WSLV known to occur in South Africa, causing sporadic outbreaks usually associated with good rainfall favouring mosquito breeding. Infectious virus can only be handled within a biosafety level (BSL) 3 facility, hence we opted to validate the assay with transcribed RNA. Specific RT-RPA primers and probes were designed for detection of WNV and WSLV and products detected using a rapid lateral flow device. The assay was performed in 30 min and detected 1.9×10^{1} copies of WNV and $3.5 \times 10^{\circ}$ copies WSLV using noninfectious transcribed RNA controls. In addition, the assay was not inhibited by the presence of mosquito extracts in spiked samples. Mismatches between the WNV and WSLV probes and other flaviviruses will likely prevent cross reactivity. The sensitivity, low RPA incubation temperature and rapid processing time makes assay systems based on RPA technology ideally suited for fieldable diagnostics.

Keywords: Flaviviruses; Isothermal RT-RPA; Transcribed RNA controls

1. Introduction

West Nile virus (WNV) and Wesselsbron virus (WSLV) are mosquito-borne viruses belonging to the *Flavivirus* genus in the family *Flaviviridae* and are the two most frequently identified flaviviruses in southern Africa. Members of the genus have a positive stranded RNA genome of approximately 11,000 nucleotides in length that encode for structural (envelope) and non-structural proteins. The non-structural 5 (NS5) protein plays a role in flavivirus replication. Regions of the gene have high similarity and are reasonably well conserved throughout the flavivirus genus. Hence the conserved regions of this gene are frequently targeted for flavivirus multiplex assays (Danecek and Schein, 2010; Fajardo et al., 2020; Johnson et al., 2010; Karothia et al., 2018; Scaramozzino et al., 2001; Sekaran and Artsob, 2007).

WNV and WSLV cause sporadic outbreaks of disease with public and veterinary health implications. Other mosquito-borne flaviviruses that have previously been identified in South Africa (SA) with potential public health implications include Spondweni virus (SPOV), Banzi virus (BANV) and Usutu virus (USUV), (Burt et al., 2014; Kokernot et al., 1957; Parkash et al., 2019; Smithburn et al., 1959; Venter et al., 2010, 2017; Weyer et al., 2013; Williams and Woodall, 1964). Dengue virus (DENV) and yellow fever virus (YFV) are not endemic to southern Africa, but are pathogenically significant with the potential to be introduced to this region.

Arboviral infections frequently present with similar clinical symptoms thus laboratory confirmation is essential. During the acute phase of illness viral infections can be detected using virus isolation, antigen detection or amplification of viral RNA using RT-PCR. Virus isolation is the reference method for arboviruses, and flavivirus diagnosis, however, isolating the virus requires high biocontainment laboratories and can take up to seven days for isolation in cells or a suitable animal model (Abd El Wahed et al., 2015). Antigen detection is frequently not considered a sensitive assay during the acute phase of illness and is less significant than a diagnosic assay. Hence molecular nucleic acid amplification assays, which are sensitive and rapid, are regarded as the most useful and frequently used assays. In instances where the period of viremia is of short duration, detection of IgG and/or IgM antibody can provide evidence of infection. Seroconversion, increasing IgG antibody titers or detection of IgM antibody responses suggest recent infection (Burt et al., 2014).

In addition, molecular assays such as conventional and real time RT-PCR techniques, can be used for surveillance of insect vectors. These techniques require thermal cyclers or more sophisticated cyclers for performing RT-PCR in real time. In contrast, isothermal molecular detection assays are relatively inexpensive alternatives to conventional and real time molecular detection methods with reportedly comparable sensitivity and specificity (Abd El Wahed et al., 2015). Isothermal assays have the advantage of potential application in field situations and could be useful for mosquito identification during outbreaks, surveillance or detection in lower income countries. There are a number of isothermal assays that have been developed including reverse-transcriptase recombinase polymerase amplification (RT-RPA) assays which can be coupled with lateral flow strips for quick point-of-care detection of products (Daher et al., 2016; Lobato and O'Sullivan, 2018). RT-RPA employs a combination of a reverse transcriptase enzyme, a recombinase enzyme, a DNA polymerase

enzyme and single-stranded DNA-binding proteins. There is no requirement for temperature cycling, and amplification generally occurs within 3−5 min. RT-RPA assays have shown to be highly tolerant of inhibitors within patient samples or crude vector preparations and have previously been described for arboviruses such as YFV and Zika virus (ZIKV) with high sensitivity and specificity, and good concordance with existing molecular assays (Chan et al., 2016; Escadafal et al., 2014; Wand et al., 2018). The ability of these assays to tolerate crude extraction procedures, such as heat based lysis methods with addition of RNase inhibitors, makes them a favourable option for field work and mosquito surveillance (Qian et al., 2020). Development of rapid low-cost molecular assays would facilitate detection of these pathogens without requirement for culture and handling of infectious material, however validation of assays requires appropriate controls and due to biosafety restrictions, the preparation of RNA controls from infectious virus is prohibited in many research laboratories. This emphasizes the need for preparation of positive RNA controls which are safe to handle in resource limited settings.

In this study the aim was to develop and evaluate a RT-RPA for detection and differentiation of two flaviviruses known to occur in SA using laboratory prepared non-infectious transcribed RNA controls and to compare RT-RPA assays with a simple one step conventional RT-PCR in terms of sensitivity.

2. Materials and methods

2.1. Design of RT-RPA and RT-PCR primers

Sequence data for the NS5 gene of various flavirviruses was retrieved from GenBank and aligned uding Clustal version X. The aligned sequence data was used to identify a 414 bp conserved region for design of primers that would amplify a range of flaviviruses and for design of probes that would differentiate between WNV and WSLV, both known to occur in southern Africa.

Similarly, the aligned data was used to design primers for the RT-RPA which required a shorter target region than the RT-PCR according to TwistAmp® nfo recommendations. For the RT-RPA, the reverse primers were labelled with digoxigenin at the 5' end and the probes were designed according to TwistAmp® nfo recommendations with an internal spacer (idSp) replacing a base and a 3' phosphate blocker. Primers and probes were prepared and supplied by Integrated DNA Technologies (IDT, Iowa, United States of America (USA)).

2.2. Preparation of transcribed RNA controls

Synthetic partial genes flanked by each primer identified in the alignment and covering a 371 bp region of WNV isolate NY99 NS5 gene (Genbank accession nr: NC_009942.1) belonging to lineage 1a and a 365 bp region of WSLV NS5 gene (Genbank accession nr: JN226796.1) were synthesized and supplied in pUC57 (Genscript, Hong Kong) (Positions 1–371 and 1–365 respectively in alignment in Supplemental data Fig. S1). Each plasmid was used to transform *Escherichia coli* (*E. coli*) JM109 competent cells (Promega, Wisconsin, USA) according to manufacturer's instructions. Positive transformants were identified via restriction enzyme digestion. The partial NS5 genes were each subsequently amplified using

GoTaq® DNA Polymerase (Promega, Wisconsin, USA) according to the manufacturer's instructions and FlaviF1 and FlaviR2 primer pair. The PCR reactions were set up as follows: initial denaturation at 95 °C for 2 min; 30 cycles of 95 °C for 30 s, 44 °C for 30 s and 72 °C for 1 min and a final extension step at 72 °C for 5 min. Purified PCR amplicons were ligated into pGEM®-T Easy vector (Promega, Madison, USA) using TA cloning. Partial NS5 gene (353 bp) amplified from South African isolate WNV SPU 93/01 lineage 2 was cloned into pGEM®T Easy. The ligated fragments were confirmed by restriction endonuclease digestion using *Not*I (Promega, Wisconsin, USA).

Templates for RNA transcription were prepared from each construct using GoTaq® DNA Polymerase according to the manufacturer's instructions and the FlaviF1 forward primer and a primer targeting the Sp6 site (5' ATTTAGGTGACACTATAG 3') on the plasmid downstream of the flavivirus reverse primer site. PCR amplicons were purified from 1% agarose gel using SV Wizard PCR Clean up kit (Promega, Madison, USA), confirmed by sequence analysis, and RNA transcribed from each template using the MEGAscript™ Sp6 Kit (Invitrogen, Massachusetts, USA) according to manufacturer's instructions.

The RNA transcripts were generated form the Sp6 promoter site on the pGEM®-T Easy plasmids and hence were 470 bp, 452 bp and 464 bp in length for WNV lineage 1a, WNV lineage 2 and WSLV, respectively. The RNA transcripts were purified using SV Total RNA Isolation System (Promega, Madison, USA) and treated with RQ1 RNase-free DNase (Promega, Madison, USA) to remove any excess DNA.

2.3. RT-PCR

Purified RNA transcripts were used to optimize a one-step RT-PCR using One*Taq*® One-step RT-PCR kit (New England Biolabs, Massachusetts, USA) and primers FlaviF1 and FlaviR1 (Table 1).

Table 1. RT-PCR reaction components per tube.

Components	Volume (µl)	Final concentration
One*Taq*® One-Step Reaction mix, 2X	12.5	1x
One*Taq*® One-Step Enzyme mix, 25X	1	1x
Flavi F1 (10 µM/µl)	1	400 nM
Flavi R2 (10 µM/µl)	1	400 nM
NFW	3,5	–
RNA	1	–
Total	25	–

The reaction was cycled as follows: reverse transcription step at 48 °C for ten minutes followed by 94 °C for one minute. Amplifications were cycled 40 times at 94 °C for 15 s, annealing temperature 44 °C for 30 s and extension for 30 s at 68 °C. A final extension step was performed for 5 min at 68 °C and held at 4 °C indefinitely. The PCR products were visualised after electrophoresis on a 1 % agarose gel.

2.4. RT-RPA test

RT-RPA was performed using transcribed RNA controls and the TwistAmp® nfo kit (TwistAmp, Cambridge, United Kingdom (UK)) according to manufacturer's instructions. M-MLV Reverse Transcriptase (Thermo Fisher Scientific, Waltham, USA) and RNaseOUT™ Recombinant Ribonuclease Inhibitor (Thermo Fisher Scientific, Waltham, USA) were included in each reaction. Optimal conditions were determined using a range of primer concentrations, incubation times and reaction temperatures, with final concentrations of 420 nM for each primer and 120 nM of the probe. (Table 2)

Table 2. RT-RPA components per reaction.

Reagent (Stock concentration)	Volume (µl)	Final concentration
FlaviF1 RT-RPA F (10 µM)	2.1	420 nM
Reverse primer[a] (10 µM)	2.1	420 nM
RPA Probe[b] (10 µM)	0.6	120 nM
Rehydration buffer	29.5	–
M-MLV reverse transcriptase (20 U/µl)	1	0.4 U/µl
RNase inhibitor (20 U/µl)	1	0.4 U/µl
RNase-free water	6.2	–
Template[c]	5	–
MgAcO (280 mM)	2.5	14 mM
Total	50	

[a]WNV RT-RPA R or WESS RT-RPA R.
[b]WNV RT-RPA probe, WNV (SA) RT-RPA probe or WESS RT-RPA probe.
[c]Transcribed RNA controls for WNV or WSLV.

The reaction mixture was added to the freeze-dried reagent pellet and incubated at 40 °C for 20 min. PCRD nucleic acid detection strips (Abingdon Health, York, UK) were used to visualize the products. Briefly, a 1:14 dilution of the reaction mix was performed (6 µL reaction sample plus 84 µL of PCRD extraction buffer) and a 75 µL aliquot of the diluted reaction mixture was added to the sample well of each PCRD test cassette. The cassette was placed in a horizontal position for 10 min, after which results were visualized. Thus, the RT-RPA test was able to provide results in 30 min.

Positive RT-RPA amplicons were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, USA), visualised after electrophoresis on a 1 % agarose gel and confirmed by nucleotide sequencing. Editing of sequence data was performed using Chromas Pro version 1.6. (Technelysium, Brisbane, Australia). The sensitivity of each RT-RPA was evaluated by testing ten-fold dilutions of transcribed RNA controls. The results were compared to a conventional RT-PCR.

2.5. Specificity and cross-reactivity of RT-RPA assays

The specificity of each RT-RPA primer pair and probe was tested using transcribed RNA for WNV, and WSLV, RNA from YFV 17D, and two unrelated arthropod-borne viruses, Sindbis virus (SINV, SAAR86) and Crimean-Congo hemorrhagic fever orthonairovirus (CCHFV). RT-

RPA assays were performed as described in section 2.3. Each RNA species included for specificity was previously verified using virus specific primers.

In addition, alignments of sequence data for dengue virus serotype 2 (DENV-2), ZIKV, Japanese encephalitis virus (JEV), YFV, Kadam virus (KADV), Langat virus (LGTV), Louping Ill virus (LIV), Royal Farm virus (RFV) and tick-borne encephalitis virus (TBEV), were considered to determine potential cross reactivities using sequence data retrieved from GenBank (Table 3).

Table 3. GenBank data for other flaviviruses.

Virus	Strain/Isolate	Country	GenBank accession number
ZIKV	PF13/251013–18	French Polynesia	KX369547.2
DENV-2	TSV01	Australia	AY037116.1
YFV	Uganda 2010	Uganda	JN620362.1
JEV	Vellore P20778	India	AF080251.1
KADV	N/A	Uganda	NC_033724.1
LGTV	TP21	Malaysia	EU790644.1
LIV	369/T2	UK	NC_001809.1
RFV	N/A	Afghanistan	NC_039219.1
TBEV	N/A	Austria	NC_001672.1
WNV	NY99 (lineage 1a)	USA	NC_009942.1
WNV	SA93/01 (lineage 2)	SA	EF429198.1
USUV	Vienna 2001	Austria	NC_006551.1
USUV	SAAR-1776	SA	AY453412.1
WSLV	AV259	SA	JN226796.1

N/A: not available.

2.6. Inhibitory effects of interfering agents in mosquito extracts

The RT-RPA was designed to have application in screening wild caught mosquitoes. To confirm that RNA extracted from mosquito pool homogenates was detectable without inhibition of the amplification, RNA spiked mosquito pools were tested. Briefly, RNA was available that had previously been extracted from pools of wild caught mosquitoes, (*Culex* mosquitoes). The mosquitoes were previously confirmed negative for WNV and WSLV using real time RT-PCR. Two pools were individually spiked with 1 µL transcribed WNV or WSLV RNA and tested using the RT-RPA and relevant primer pair and probe.

3. Results

3.1. RT-RPA and RT-PCR assay design

The RT-RPA and RT-PCR primers were designed using alignments of the partial NS5 gene of 16 flaviviruses representing viruses from different geographic regions and including flaviviruses known to occur in southern Africa (Supplemental data Table S1). A degenerate

Table 4. RT-RPA primers and probe.

Primer name	Virus	Genomic positions on reference strains[a]	Oligonucleotide sequence (5' to 3')	%GC content	Tm (ºC)	Size of primer or probe (nt)
FlaviF1 RT-RPA F	**WNV, WSLV**	8707–8723* **8639–8655****	ATGGC*H*ATGAC*W*GACAC[b]	49	50.1	17
WNV RT-RPA R	WNV	9059–9031	/DigN[c]/CTCTTTCCCATCATGTTGTAAATGCAAGT	37.9	57.8	29
WNV RT-RPA Probe 1	WNV	8908–8954	/Biosg[d]/GCTTTGGGTGCCATGTTTGAAGAGCAGAATC/idSp[e]/ATGGAGGAGCGCCAG/Phos[f]/	54.3	70.5	47
WNV RT-RPA Probe 2	WNV	8731–8776	/Biosg[d]/TTCGGTCAACAACGAGTGTTCAAGGAAAAG/idSp[e]/TGGACACAAAGGCTC/Phos[f]/	48	69	45
WESS RT-RPA R	WSLV	8991–8962	/DigN[c]/CGTTTTCCCATCATGTTGTACACACATGTC	59.8	43.3	30
WESS RT-RPA Probe	WSLV	8875–8921	/Biosg[d]/GAAGAGTGCAAGTGAGGCTGTTCAGGATCC/idSp[e]/CAGTTCTGGAAACTGG/Phos[f]/	52.2	68.8	47

[a]WNV reference isolate (*GenBank accession number: NC_009942.1), WSLV isolate AV259 (**GenBank Accession number: JN226796.1).
[b]Wobble bases represented as follows ***H***: A or C or T; ***W***: A or T.
[c]DigN = Digoxigenin label.
[d]Biosg = Biotin label.
[e]idSp = Internal spacer.
[f]Phos = Phosphate blocker.

primer pair designated FlaviF1 and FlaviR1 was identified which amplified a 353 bp region (Supplemental data Fig. S1). The primers for the RT-PCR were designed to target multiple flaviviruses so that future studies could accommodate emerging or imported flaviviruses using the same region, primers and transcribed RNA controls. (Table 4). The primers and probes for the RT-RPA assay were based on amplification of WNV and WSLV both known to occur in SA.

Two reverse primers were necessary for the RT-RPA to accommodate mismatches in the selected region and to ensure the recommended length for the assay. In contrast, only one reverse primer was designed for the RT-PCR in the same region, however, as it was shorter it covered a conserved region with fewer mismatches to accomodate. Two probes were designed for WNV due to mismatches between lineage 1a and lineage 2.

To compare the RT-RPA with a conventional RT-PCR that could be performed within a similar time frame and without the need for expensive real-time cyclers, in-house degenerate primers designated FlaviF1 and FlaviR2 targeting a 350 bp region of the NS5 gene were designed (Table 5).

Table 5. RT-PCR primers.

Primer name	Genomic positions on reference strains[a]	Oligonucleotide sequence[b] (5' to 3')	%GC content	Tm (ºC)	Length of primer (bp)
FlaviF1	8707–8723 (NC_009942.1; 8639–8655 (JN226796.1)	ATGGC*H*ATGAC*W*GACAC	49	50.1	17
FlaviR2	9059–9040 (NC_009942.1); 8991–8972 (JN226796.1)	C*YN*TT*Y*CCCATCATGTT*N*TA	37.5	44	20

[a]WNV reference isolate (GenBank accession number: NC_009942.1), WSLV isolate AV259 (Accession number: JN226796.1).
[b]Wobble bases represented as follows ***H***: A or C or T; ***W***: A or T; ***Y***: C or T; ***N***: any base.

3.2. Transcribed RNA controls

Flaviviruses with public and veterinary health implications require BSL 3 or higher facilities, hence we elected to prepare synthetic RNA controls for validation of the assays. Three controls were prepared to represent WNV lineage 2, WNV lineage 1a, and WSLV. All transcribed RNA preparations were screened using PCR to confirm adequate digestion of DNA template. Serial dilutions of RNA were used to determine minimum detection levels. In addition, RT-RPA amplicons detected using the LFA were sequenced using Sanger sequencing. Nucleotide sequence data was edited using Chromas Pro version 1.6 and the viral identities were determined by comparison with nucleotide sequence data retrieved from GenBank and with Basic Local Alignmnet Search Tool (BLAST) analysis. There was 100 % concordance between sequence data obtained and data used to prepare synthetic genes.

3.3. RT-PCR and RT-RPA

WNV and WSLV were amplified using a conventional RT-PCR with visualisation of stained DNA amplicons after electrophoresis using 1 % agarose gel and a gel imager. In comparison,

the RT-RPA was visualised using lateral flow assays which minimized requirements for electrophoresis equipment and imager. WNV and WSLV RNA controls were detected after amplification using the lateral flow cassettes (Fig. 1). Each cassette has three reaction lines – line 1 for detection of DIG/Biotin-labelled amplicons; line 2 for detection of FAM/Biotin- or FITC/Biotin-labelled amplicons and line C is the flow-check control line. Digoxigenin-labelled products reacted at the position of line 1.

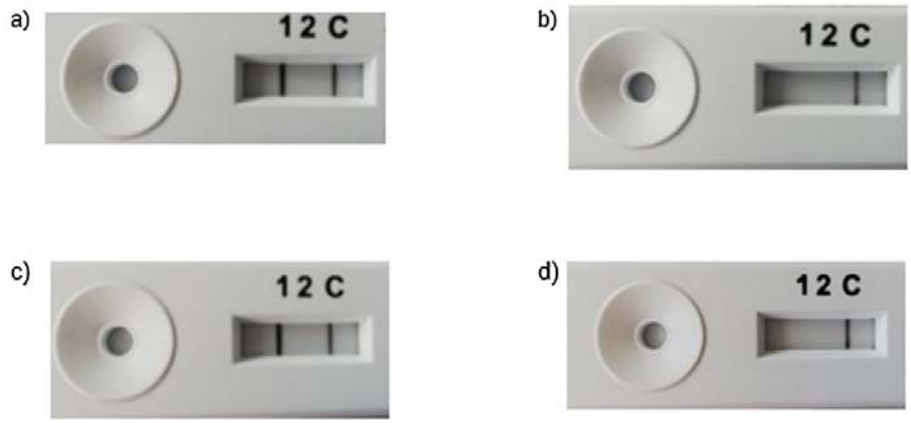

Fig. 1. RT-RPA products were visualised using lateral flow assays. a) WNV RT-RPA, b) Negative control for WNV RT-RPA, c) WSLV RT-RPA, and d) Negative control for WSLV RT-RPA.

3.4. Sensitivity of RT-RPA

To determine the sensitivity of the RT-RPA ten-fold dilutions of each flavivirus RNA control were tested. The results were compared with a conventional flavivirus RT-PCR in which only one round of reaction was performed. Although the sensitivity of the conventional RT-PCR could likely be improved using a nested reaction or a real-time reaction, the aim was to compare the RT-RPA with a simple molecular assay that could be performed in a similar time frame and without expensive equipment such as a real-time cycler. The RT-RPA detected 1.9×10^0 and 3.5×10^1 RNA copies of WNV lineage 2 and WSLV, respectively. In comparison the conventional one round RT-PCR detected 1.9×10^4 and 3.5×10^5 RNA copies of WNV lineage 2 and WSLV, respectively.

3.5. Specificity of RT-RPA

It was anticipated that the assay would be able to differentiate between WNV and WSLV and this was shown to be applicable. In addition, RNA from other arboviruses known to occur in SA (SINV and CCHFV) or previously imported in travellers (YFV) were tested using the RT-RPA and were all negative.

To further investigate the possible cross-reactivity for RNA species not available in our laboratory, sequence data from the flavivirus multiple alignment of the partial NS5 region (Supplemental data Fig. S1 and Table S1) was considered for similarity or mismatches. Potential cross-reactivities were determined through comparison with the sequence of the partial NS5 region of YFV, ZIKV, DENV-2, JEV, KADV, LGTV, LIV, RFV and TBEV by determining number of mismatched bases (Table 6).

Table 6. Observed mismatches in the multiple alignments of selected flavirviruses.

	Primer / probe				
	WNV RT-RPA probe 1	WNV RT-RPA R	WNV RT-RPA probe 2	WESS RT-RPA probe	WESS RT-RPA R
Flaviviruses	**Number of mismatches (bp)**				
DENV-2	18	4	13	13	2
JEV	14	4	9	14	4
KADV	11	8	7	12	7
LGTV	16	7	12	11	6
LIV	16	8	12	17	10
RFV	12	7	10	15	7
TBEV	16	6	9	13	7
YFV	18	4	14	10	4
ZIKV	15	6	10	17	4
USUV	4	3	11	10	6
USUV (SAAR-1776)	6	3	10	10	6
WNV (NY99)	**0**	**0**	7	14	7
WNV (SA93/01)	10	**2**	**0**	13	7
WSLV (AV259)	16	6	11	**0**	**0**
WSLV (SAH-177)	16	7	11	**1**	**1**

The cells of each primer or probe used for a homologous virus are highlighted in grey.

3.6. Inhibitory effects of interfering agents in mosquito extracts

Isothermal methodologies such as RT-RPA are usually tolerant to crude samples and allow detection in arthropod preparations with minimal processing (Bonney et al., 2017). In the absence of WNV or WSLV positive mosquito pools in our laboratory, the potential inhibitory effect of interfering agents within a crude mosquito sample was tested by spiking two mosquito pools that previously tested negative for WNV and WSLV RNA using real-time qRT-PCR in a previous study. Each pool was spiked with 1 µL transcribed RNA and the RT-RPA reactions using both WNV and WSLV primers and probes were positive, suggesting no inhibition of the reaction from the mosquito homogenate.

4. Discussion

Molecular assays are frequently used as diagnostic or surveillance tools. Some assays require expensive equipment and skilled expertise and are less suitable for low resource countries, hampering surveillance in these areas. Surveillance of mosquito populations as well as sentinel animals, such as horses, for arboviral infections could play an important role in early detection of arbovirus outbreaks. There has been a proliferation of molecular assays that can be easily adapted for field settings (Bonney et al., 2017), and these could be useful for countries and/or laboratories with limited resources and where surveillance would play a role in identifying which flaviviruses are circulating. Similarly, reasonably priced assays should enhance diagnostic availability for humans and for animals, particularly horses that are susceptible to severe and fatal infections. Real time and conventional RT-PCR are

frequently used to detect flaviviruses but do require sophisticated equipment and reagents (Escadafal et al., 2014). RPA is becoming a molecular assay of choice for the rapid, specific and sensitive identification of pathogens, even as a fieldable diagnostic (Daher et al., 2016). Positive controls are essential components to validate any assay. A positive control needs to contain the target gene and must amplify using the same conditions as test samples. In this study we focused on WNV and WSLV, two arboviruses known to occur in SA that are difficult to differentiate clinically and probably both are under reported. Serological assays will likely not differentiate between these viruses hence molecular assays have an important role. In this study synthetic genes and a PCR amplicon were used to prepare RNA controls, avoiding the need to handle biohazardous material. Transcribed RNA was simple to prepare and allowed sensitivity assays to be performed to determine minimum detection levels of RNA. Hence an isothermal assay using non-infectious RNA controls could be an ideal assay for field studies, low-income regions or remote areas.

In previous reports, a RT-RPA designed for the rapid and sensitive detection of ZIKV was able to provide results available in less than 15 min (Chan et al., 2016). The assay described in this study required 30 min to obtain a result, in comparison the RT-PCR required at least 3–4 hours to obtain a result. Two RT-RPA assays have been described for the detection of DENV 1–4, and using RNA molecular standards, an analytical sensitivity ranging from 14 (DENV 4) to 241 (DENV 1–3) RNA copies was detected (Abd El Wahed et al., 2015). To investigate the feasibility of developing a RT-RPA for flaviviruses, RT-RPA assays were investigated for the detection of two flaviviruses, WNV and WSLV, which are known to occur in SA. Sequence data for selected mosquito and tick borne flaviviruses were aligned, including WNV, WSLV, DENV-2, YFV, ZIKV, USUV, TBEV, LGTV, LIV, RFV, KADV and JEV. Based on this alignment, a 414 bp region of the partial NS5 gene was identified as one of the most conserved regions of the genome and a pair of degenerate RT-PCR consensus primers were identified that targeted this region and hence the region was selected as a target for the RT-RPA.

The RT-PCR primers, FlaviF1 and FlaviR2, were designed as degenerate primers to amplify WNV and WSLV RNA, and were successfully able to amplify the transcribed RNA. The RT-RPA and detection of product using an LFA was more sensitive than the RT-PCR. A nested conventional RT-PCR would certainly improve the sensitivity of this assay, however all we aimed to do in this study was determine if the RT-RPA with rapid detection of amplification products was suitable as a quick assay. In addition, testing RNA from other arboviruses suggested that despite the binding tolerability of the assay there was good specificity. This was in agreement with RPAs developed for the detection of other pathogens like Ebola virus, Sudan virus, *Yersinia pestis* and *Bacillus anthrax* where high specificity has been described (Euler et al., 2013). WNV and WSLV only reacted with homologous probes, suggesting that performing two assays namely WNV and WSLV, should suffice to detect and differentiate the flaviviruses. The number of mismatches between the WNV and WSLV probes suggest it is theoretically unlikely that these primer pairs and probes would amplify other known flavivirures except possibly USUV. Currently USUV is not known to be circulating in SA, although historically it has been detected in the country (Burt et al., 2014), hence this needs to be considered. USUV and WNV share similar vector mosquito species, hence it is possible that USUV could re-emerge in the country. Sequencing of amplicons would be required for differentiation.

In this study primers and probes were designed specifically for WNV lineage 1a and lineage 2. Nine lineages of WNV have been proposed, however most severe cases of human and equine disease are associated with lineages 1 and 2. Lineage 1 is globally widespread in areas where WNV circulates whereas Lineage 2 was previously only considered to circulate in SA but has recently emerged in countries in Europe and lineage 1 has been identified in SA. (Mackenzie and Williams, 2009; Pachler et al., 2014). Hence, we selected to include both lineages in this study.

In conclusion, the RT-RPA proved to be a robust, sensitive and specific assay that is not time consuming and can be used as a possible fieldable diagnostic or in surveillance studies.

Authors' contributions

E Bonnet: Investigation, Validation, Formal analysis, Writing – Original draft, Visualization

D van Jaarsveldt: Investigation, Formal analysis, Writing – Original draft.

FJ Burt: Conceptualization, Methodology, Writing – Review & Editing, Supervision, Project administration, Funding acquisition.

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Declaration of Competing Interest

The authors declare that they have no conflicting interest.

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