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Development and analytical validation of a group-specific RT-qPCR assay for the detection of the Simbu serogroup orthobunyaviruses

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Highlights

- A group-specific RT-qPCR assay was developed for Simbu serogroup orthobunyaviruses.
- The assay can discriminate phylogenetic clades A and B.
- The assay was developed using all publically available sequencing data.
- The assay is efficient, sensitive and specific.

Abstract

The Simbu serogroup within the genus Orthobunyavirus belongs to the family Peribunyaviridae and comprises 32 recognised three-segmented negative-sense single-stranded RNA viruses, with a cosmopolitan distribution. This group of arthropod-borne viruses includes important pathogens of humans and domestic animals e.g. Oropouche

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orthobunyavirus and Schmallenberg virus. Sensitive and specific diagnostic tools are required for recognition and control of outbreaks. A novel TaqMan® RT-qPCR assay was developed, optimised and analytically validated for the broad detection of the Simbu serogroup orthobunyaviruses.

A region in the S segment, which encodes the nucleocapsid protein, was used to design a group primer set and a pair of differently labelled TaqMan® minor groove binder probes to distinguish phylogenetic clade A and B of the serogroup. Efficiencies determined for seven members of the group were 99% for Akabane orthobunyavirus (AKAV), 96% for Simbu orthobunyavirus (SIMV), 96% for Shuni orthobunyavirus (SHUV), 97% for Sathuperi orthobunyavirus (SATV), 84% for Shamonda orthobunyavirus (SHAV), 93% for Ingwavuma virus (INGV, now classified as Manzanilla orthobunyavirus) and 110% for Sabo virus (SABOV, now classified as AKAV). The 95% limit of detection (TCID50/reaction) was 10-3.61 for AKAV, 10-2.38 for SIMV, 10-3.42 for SHUV, 10-3.32 for SATV, 10-1.67 for SHAV, 100.39 for INGV and 10-2.70 for SABOV.

Keywords

Simbu serogroup viruses, Clade A, Clade B, TaqMan®, real-time RT-PCR

1. Introduction

The genus Orthobunyavirus belongs to the family Peribunyaviridae, order Bunyavirales, and currently comprises 49 species of viruses, represented by approximately 170 exemplar isolates (Adams et al., 2017). Historically, the viruses were classified into 18 serogroups based on their antigenic relationships in serological assays, such as haemagglutination inhibition (HI), complement fixation (CF) and serum neutralisation (SN) tests (Calisher, 1983; Chowdhary et al., 2012; Elliott and Blakqori, 2011; Gauci et al., 2015; Kinney and Calisher, 1981; Travassos da Rosa et al., 1983).

Bunyaviruses are characterised by having three single-stranded negative-sense RNA genomic segments, designated large (L), medium (M) and small (S), of approximately 6.9 kb, 4.5 kb and 1.0 kb. The segments are translated into six proteins (Elliot, 1990), namely viral RNA-dependent RNA polymerase (RdRp), encoded in the L segment, the surface glycoproteins, Gn and Gc, encoded in the M segment, the nucleocapsid protein (N), encoded in the S segment, as well as two non-structural proteins, NSm and NSs, encoded in the M and S segments (Bishop et al., 1980; Elliott, 2014; Elliott and Blakqori, 2011; Saeed et al., 2001).

The Simbu serogroup, named after the prototype virus, is the largest of the serogroups and comprises highly diverse arboviruses (Saeed et al., 2001). The concept of serogroup played an important role in the development of arbovirus taxonomy (Casals, 1957; Calisher and Karabatsos, 1988), but is no longer used by the International Committee on Taxonomy of Viruses (ICTV), as comparative analysis of nucleic acid and protein sequences has become

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the primary method for determining virus relationships (Saeed et al., 2001). Due to the lack of genetic data, most current taxonomic assignments are still based on serology (Adams et al., 2017; Nichol et al., 2005; Plyusnin et al., 2012).

At present, the Simbu serogroup comprises 32 virus isolates, of which 28 have been subjected to full genome sequencing (Table 1) (Ladner et al., 2014; Saeed et al., 2001; Tilston-Lunel et al., 2015; Zhang et al., 2015). Members of the group have recently been assigned to eight species, based on close antigenic relationships and less than 10% differences in amino acid sequences of the nucleocapsid protein between species (Table 1) (ICTV, 2015).

The Simbu serogroup is divided into two phylogenetic clades, designated A, those known to affect the central nervous system in humans (Aguilar et al., 2011; Anderson et al., 1961; Ladner et al., 2014), and B, those associated with abortion, teratology and encephalitis in ruminants, as well as neurologic disease in horses (Table 1) (Charles, 1994; Coetzer and Howell, 1998; Coverdale et al., 1978; Hirashima et al., 2017; Hoffmann et al., 2012; Kono et al., 2008; Miyazato et al., 1989; van Eeden et al., 2012). The viruses have also been found in a wide range of wild mammals and birds (Anderson et al., 1960; Calisher et al., 1969; Carey et al., 1971; McIntosh et al., 1965; Navarro et al., 2016; Pajot, 1980; Reeves et al., 1970; Seymour et al., 1983) and invertebrate hosts (Cybinski, 1984; De Regge et al., 2012; Lee et al., 1979; Reeves et al., 1970; St. George et al., 1980; Sandfast and Dyce, 1982).

The Simbu serogroup has a cosmopolitan distribution. Clade A comprises mainly viruses that occur in the Americas (but including Facey's Paddock virus from Australia, Cat Que virus from Asia, and Ingwavuma virus that occurs in Africa and Asia). Clade B members are distributed widely in Africa, Asia and Oceania, with a few individual viruses occurring in all three regions. The recent discovery of Schmallenberg virus (SBV) extends the known distribution of the clade into Europe (Table 1). Leanyer virus (LEAV), of Australia, does not fall into either of the two clades.

Globalisation, alongside climate, ecological, demographic and socioeconomic changes are contributing to the emergence of pathogenic viruses (Lipkin, 2013; Vasconcelos et al., 2001), and orthobunyaviruses are considered to be underestimated with regard to prevalence, distribution and disease associations (Weidmann et al., 2003).

Molecular assays have been developed for the detection of Simbu serogroup orthobunyaviruses, involving either virus-specific approaches in a simplex format (Bilk et al., 2012; Tauscher et al., 2017; Van Eeden, Zaayman and Venter, 2014), or broader approaches in duplex (Stram et al., 2004) and multiplex formats (Fischer et al., 2013; Golender et al. 2018; Lee et al., 2015; Naveca et al., 2017; Shirafuji et al., 2015). Most assays target the S segment, the least variable segment (Acrani et al., 2010; Cardoso et al., 2015; Hang et al., 2014; Nunes et al., 2005; Saeed et al., 2000; Vasconcelos et al., 2011), but an assay targeting the L segment of a broad-range of Simbu serogroup viruses has been described (Fischer et

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al., 2013). Other molecular assays with broad detection capacity include Shirafuji et al. (2015), who used TaqMan® chemistry for the detection of clade B viruses, and recently, a new TaqMan® based assay detected eleven clade B isolates (Golender et al. 2018).

We describe a novel RT-qPCR assay utilising TaqMan® minor groove binder (MGB) probes for the broad detection of Simbu serogTroup viruses and discrimination between clades A and B. All available Simbu serogroup genomic data were evaluated to design the assay. The fitness of purpose of the assay is to determine freedom of infection, to confirm diagnosis of suspect or clinical cases and to estimate the prevalence of infection and facilitate risk analysis.

2. Materials and methods

2.1. Simbu serogroup isolates

Simbu serogroup orthobunyaviruses stored in the Department of Veterinary Tropical Diseases (DVTD), Faculty of Veterinary Sciences, University of Pretoria were used for the development and optimisation of the assay, namely Akabane orthobunyavirus (AKAV), Simbu orthobunyavirus (SIMV), Shuni orthobunyavirus (SHUV), Sathuperi orthobunyavirus (SATV), Shamonda orthobunyavirus (SHAV), Ingwavuma virus (INGV, now classified as Manzanilla orthobunyavirus) and Sabo virus (SABOV, now classified as AKAV) (Table 1). The viruses were originally identified by means of complement fixation and neutralization tests (Costa Mendes, 1984), and identities confirmed for the present study with an existing pan-Simbu RT-qPCR assay (Fischer et al., 2013).

2.2. Virus titration

African green monkey kidney (Vero) cells (ATCC®) were grown in minimum essential medium (MEM) with Earle's balanced salt solution supplemented with 5% γ-irradiated foetal bovine serum (FBS) (Biowest, Denmark) and 50 mg/L gentamicin (Virbac, South Africa) (MEM+), at 37°C in a humidified 5% CO2 atmosphere. Five replicates of 100 µl inoculum per dilution from 10-1 to 10-8 of the test viruses were added per well to cell cultures in 96-well microplates (Thermo Fisher Scientific, USA). Cytopathic effect (CPE) was read after six days incubation, and 50% tissue culture infectious dose (TCID50) end points calculated (Kärber, 1931; Spearman, 1908).

2.3. Evaluation of genome variation

Amino acid sequences from the three segments of all the Simbu serogroup orthobunyavirus sequences available in the GenBank® database were aligned online, using the default settings of MAFFT version 7.3.1.3 (Katoh et al., 2017). The EMBOSS: tranalign (Rice et al., 2000) programme was used to back-translate the aligned amino acid sequences to nucleic acid sequences. BioEdit Sequence Alignment Editor version 7.2.3 (Hall, 1999) was used to edit the sequences and to visualise conserved regions in the open reading frame (ORF).

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DAMBE software package version 5.3.48 (Xia, 2013) was used to identify identical sequences. To visualise the nucleotide variation along the three segments, a variation score was developed and calculated using the following equation: variation score = $1 - x1 + x2 + 2x3 + 3x4$, where xi = frequency per nucleotide position of the most common nucleotides ($1 \leq i \leq 4$) ranked from most common (1) to least common (4). The variation score was plotted against nucleotide position.

2.4. Nucleic acid purification

The nucleic acid in samples was purified with a magnetic bead-based separation method, using 50 µl sample and the MagMAX™ Pathogen RNA/DNA Kit (Thermo Fisher Scientific, USA), according with the manufacturer's instructions. The purification was automated using the KingFisher™ Duo Prime Purification System (Thermo Fisher Scientific, USA) and a standard protocol (MagMAX_Pathogen_Stnd_Vol_DUO.bdz). The nucleic acid was eluted in 50 µl Elution Buffer.

2.5. Sequencing

Seven primer sets were designed for the sequencing of the S segment of the DVTD strains: Ja_GAr_39 (AKAV), SA_Ar_53 (SIMV), An_10107 (SHUV), IG_10310 (SATV), An_5550 (SHAV), SA_An_4165 (INGV) and An_9398 (SABOV), as well as two other South African strains: 8912 (SHUV) and Cu_1/70 (SABOV) (Table 2).

The primers were designed using the PrimerQuest® tool (Integrated DNA Technologies, Inc., USA) and synthesised by Integrated DNA Technologies, Inc., re-suspended in Tris-EDTA (TE) buffer to a 100 µM stock concentration and working aliquots at 20 µM were produced by dilution with ultrapure 18.2 MΩ.cm 25°C water (Elix® Essential 5 and Synergy® water purification systems, Merck, USA).

A conventional RT-PCR was conducted utilising the SuperScript® III One-Step RT-PCR System with Platinum® Taq DNA Polymerase protocol (Thermo Fisher Scientific, USA), which consisted of a 50 µl total reaction volume composed of 25 µl 2X Master Mix, 2 µl SuperScript® III RT/Platinum® Taq Mix, 0.5 µl of each 20 µM primer, 2 µl RNA template and nuclease-free water to make up the final volume. The reactions were performed in a Veriti™ 96-Well Fast Thermal Cycler (Thermo Fisher Scientific, USA) and the reaction conditions were 55°C for 30 min, 94°C for 2 min, followed by 40 cycles of 94°C for 15 s, 55°C for 30 s and 68°C for 1 min. Gel electrophoresis was carried out with a 2% agarose gel, using 1× tris-acetate-EDTA (TAE) buffer in the presence of ethidium bromide stain. Fragment size was compared to a GeneRuler 100 bp DNA ladder (Thermo Fisher Scientific, USA) and visualised with a ChemiDoc™ XRS+ System with Image Lab™ Software version 3.0 (Bio-Rad, USA). Unincorporated nucleotides were dephosphorylated and unused primers digested with CleanSweep™ PCR purification reagent (Thermo Fisher Scientific, USA), according to the manufacturer's instructions, in a Veriti™ 96-Well Fast Thermal Cycler. Sanger sequencing

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(Sanger, Nicklen, and Coulson, 1977) was performed by Inqaba Biotec™ (Pretoria, South Africa) and the trace files edited and assembled using the Staden Software Package version 1.5 (Staden, F. Beal, and K. Bonfield, 2000).

2.6. The group-specific RT-qPCR assay

Primer Express® version 3.0.1 (Life Technologies Corp., USA) software was used to design a group-specific primer set, targeting all the Simbu serogroup orthobunyaviruses and two TaqMan® MGB hydrolysis probes (Thermo Fisher Scientific, USA). The one probe, labelled with a VIC® fluorescent dye, targeted clade A viruses, and the other probe, labelled with FAM™ (6-carboxyfluorescein) fluorescent dye, targeted clade B viruses. In silico specificity screening was carried out using the Basic Local Alignment Search Tool (BLAST®) to evaluate non-specific hybridisation. The lyophilised primers (Integrated DNA Technologies, Inc., USA) were re-suspended in TE buffer to 100 µM. Primers and probes were diluted to a 20 µM working concentration with ultrapure water.

All the reactions were set up manually in either MicroAmp™ Optical 8-Cap Strips (Thermo Fisher Scientific, USA) or MicroAmp™ Fast Optical 96-Well Reaction Plates, 0.1 mL (Thermo Fisher Scientific, USA), using the TaqMan® Fast Virus 1-Step Master Mix (Thermo Fisher Scientific, USA) protocol in a StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific, USA) running StepOne™ Software version 2.3 (Thermo Fisher Scientific, USA). Reactions consisted of 5 µl master mix, 400 nM of each primer (Simbu_F and Simbu_R), 500 nM probe Simbu_CladeAP, 250 nM probe Simbu_CladeBP, 2 µl of purified nucleic acid and H2O to make up a 20 µl total volume. The thermal-cycling conditions comprised a holding stage of 50°C for 5 min, 95°C for 20 s, followed by 40 cycles of 95°C for 3 s and 60°C for 30 s.

2.7. Optimisation

To optimise the forward and reverse primer concentration in the RT-qPCR reaction, four different primer concentrations were tested with a constant probe concentration of 250 nM: 100 nM, 200 nM, 400 nM and 800 nM. To optimise the probe concentration, different probe concentrations for both probes were tested with a constant primer concentration of 200 nM: 50 nM, 100 nM, 150 nM, 200 nM and 250 nM. Additional Simbu_CladeAP probe concentrations of 500 nM and 750 nM were also tested. Additional testing involved testing 400 nM and 800 nM primer concentrations against two different probe concentrations: 250 nM and 500 nM.

2.8. Analytical validation

2.8.1. Efficiency

The PCR efficiency was determined for the cell cultured AKAV, SIMV, SHUV, SATV, SHAV, INGV and SABOV. A ten-fold eight-log serial dilution was made for the seven viruses in nuclease-free water and each dilution was tested in triplicate. The amplification efficiency

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was calculated from the slope of the linear regression between quantification cycle (Cq) values and log TCID50/reaction using the following formula: PCR efficiency (%) = 100 × (10-1/slope −1).

2.8.2. Sensitivity

Two-fold dilution series were carried out with 10-5 AKAV, 10-5 SIMV, 10-5 SHUV, 10-5, 10-5 SATV, 10-3 SHAV, 10-3 INGV and 10-3 SABOV dilutions in nuclease-free water, to cover the non-linear range of the assay at the limit of detection. The dilution series was tested four times in a single run and the results utilised to calculate the 95% limit of detection (LOD) of the assay by probit analysis, using SPSS® Statistics version 25 (IBM®, USA). From this computer-based regression modelling, the analytical sensitivity, which is defined as the viral titre detected 95% of the time, was calculated.

2.8.3. Specificity

Probe specificity was determined for all tested viruses ensuring that Simbu_CladeAP probe does not recognise clade B isolates and vice versa.

The nucleic acid of genetically related, causative agents of abortion in ruminants and arthropod-borne viruses such as Pestivirus A (BVDV-1, V27/04 C #2 MDBK 6.12.04), Bovine alphaherpesvirus 1 (BoHV-1, #3 MDBK 12.10.00, IBR American strain), Bluetongue virus (BTV-1, pp 28.1.98), Bovine fever ephemerovirus (BEFV, #2 BHK 20.9.95, Van der Westhuizen strain), Rift Valley fever phlebovirus (RVFV, TC50+ #2 Vero 31.5.95), Wesselsbron virus (WSLV, #9 Vero 8.6.15) and Palyam virus (PALV, Kasba #1 Vero 19.2.16) were used to determine the assay specificity. In silico specificity was also performed by BLAST® analysis.

2.8.4. Repeatability

The repeatability of the assay was determined by repeating the experiment used for sensitivity determination four times, in separate runs. INGV and SATV were used as templates to represent Simbu clades A and B, respectively. The data generated were used to assess the intra-run, inter-run and overall variation for both probe Simbu_CladeAP and Simbu_CladeBP detection.

2.9. Comparison with a pan-Simbu assay (Fischer et al., 2013)

The group-specific RT-qPCR assay was compared with a pan-Simbu RT-qPCR assay (Fischer et al., 2013), by using 10-1 dilutions of AKAV, SIMV, SHUV, SATV, SHAV, INGV and SABOV. The latter assay was conducted with a reverse transcription step using the Omniscript® Reverse Transcription Kit (QIAGEN, Germany) in a 20 µl reaction volume, which comprised 2 µl 10× Buffer RT, 2 µl dNTPs mix, 2 µl 10 µM random primers, 1 µl Omniscript® RT, 2 µl template RNA and nuclease-free water. The reaction was run in a Veriti™ 96-Well Fast Thermal Cycler for 60 min at 37°C. The subsequent step involved a SYBR® Green I based

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qPCR with the KAPA SYBR FAST qPCR Master Mix (2×) ABI Prism™ (Kapa Biosystems, USA) as follows: 10 µl 2× Master Mix, 1 µl 10 µM of each primer (panOBV-L-2959 F and panOBV-L-3274R), 2 µl cDNA and nuclease-free water to a final volume of 20 µl. The real-time reaction was performed in a StepOnePlus™ Real-Time PCR System and the thermal cycling conditions consisted of a holding stage of 95°C for 20 s followed by 40 cycles of 95°C for 3 s, 55°C for 20 s and 72°C for 10 s. The melting curve was acquired with the following steps: 95°C for 15 s, 55°C for 1 min, data collection with increase of temperature at 0.3%, and 95°C for 15 s.

3. Results

3.1. Infectivity

The log TCID50/ml values obtained for the viruses cultured in Vero cells were 4.9 (AKAV), 5.7 (SIMV), 4.5 (SHUV), 5.3 (SATV), 5.3 (SHAV), 6.5 (INGV) and 4.3 (SABOV). The corresponding Cq values determined by RT-qPCR were 15.5, 15.0, 18.5, 17.0, 18.9, 25.7 and 24.7 respectively.

3.2. Evaluation of genome variation

There was very high nucleotide variation along the ORFs of all the three segments, S (n = 175), M (n = 62) and L (n = 63) (Appendix A). The 5'-end of the S segment showed the least amount of variation, which was selected as the target region of the assay we developed.

3.3. Sequencing

The S segment of the DVTD Simbu serogroup orthobunyavirus strains were all amplified successfully, with the exception of An_5550 (SHAV). Further investigation confirmed that the primer in question (Shamonda_NC018464R) was complementary with the published sequence used for its design (NC018464), but targeted a non-virus sequence that had been included in the published sequence. Successful amplification of SHAV was accomplished using the reverse primer utilised for SATV amplification (Sathuperi_HE795104R), as this was 100% identical to the SHAV sequence.

The sequences of the DVTD Simbu serogroup orthobunyavirus strains (MH999468 - MH999476) were compared with other sequences on GenBank®. Ja_GAr_39 (AKAV) showed 99% nucleotide identity to strains B8935 and R7949 (AB000853/4) and 93 - 94% nucleotide identity to other Ja_GAr_39 strains (AF034939 and AB000852). SA_Ar_53 (SIMV) was 99 - 100% identical to other published SA_Ar_53 strains (AF362397 and HE795110). The two sequenced strains of SHUV, An_10107 and 8912, were identical to each other and 99% identical to another An_10107 strain (HE800143). An_5550 (SHAV) was 100% identical to another An_5550 strain (HE795107). SA_An_4165 (INGV) was 99% identical to other SA_An_4165 strains (KF697141 and AF362395). An_9398 (SABOV) was 99 - 100% and Cu_1/70 (SABOV) 98% identical to other An_9398 strains (AF362396, HE795098). No

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reference to IG_10310 (SATV) was found in GenBank®, but it was 100% identical to HE795104, a SATV sequence published by Goller et al., (2012).

3.4. The group-specific RT-qPCR assay

A conserved region situated between nucleotide positions 120-242 (using the ORF of NC018477 for numbering) of the S segment was selected to design a group-specific primer set (Simbu_F and Simbu_R), based on the published sequencing data of twenty-eight out of thirty-two Simbu serogroup members, in order to amplify a product of 122 bp. Two TaqMan® MGB probes, one targeting clade A viruses (Simbu_CladeAP), and another targeting clade B viruses (Simbu_CladeBP) were labelled with different dyes to allow for the distinction between phylogenetic clades. Both primer and probe sets were degenerate, as this genetically diverse group of viruses presented multiple nucleotide mismatches after alignment (Table 3 and Figure 1).

The fluorescence intensity obtained from testing the Simbu serogroup clade A virus was lower compared to the testing of Simbu serogroup clade B viruses (Figure 2). This difference may be explained, possibly, by the INGV isolate used during testing, as multiple nucleotide mismatches were found between both primers and the virus.

3.5. Optimisation

The optimal concentration of primers and probes was evaluated subjectively and was judged on the lowest concentration of primers or probe able to generate a low Cq with high efficiency. The lowest Cq was obtained when using 800 nM primer concentration and no significant difference was observed between a 200 nM or 400 nM primer concentration. The detection of the target improved as the concentration of the probe increased. To limit the primer/probe concentrations in order to allow multiplex detection, a final primer concentration of 400 nM, a probe Simbu_CladeAP concentration of 500 nM and a probe Simbu_CladeBP concentration of 250 nM probe in the reaction was selected.

3.6. Analytical validation

3.6.1. Efficiency

The amplification efficiency was calculated from the linear regression between the Cq and TCID50/reaction values obtained for each isolate: 99% for AKAV, 96% for SIMV, 96% for SHUV, 97% for SATV, 84% for SHAV, 93% for INGV and 110% for SABOV (Figure 3).

3.6.2. Sensitivity

The 95% limit of detection (LOD) of the assay, expressed by TCID50/reaction, determined by probit analysis for each virus, was 10-3.61 for AKAV (95% confidence interval [CI]: 10-3.98 to 10-2.38), 10-2.38 for SIMV (95% CI: 10-2.94 to 10-0.18), 10-3.42 for SHUV (95% CI: 10-3.66 to 10-2.05), 10-3.32 for SATV (95% CI: 10-3.56 to 10-1.95), 10-1.67 for SHAV (95% CI: 10-

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2.07 to 101.57), 100.39 for INGV (95% CI: 100.03 to 101.56) and 10-2.70 for SABOV (95% CI: 10-3.01 to 10-1.59) (Fig. 4).

3.6.3. Specificity

No cross-reactivity was observed between the two probes.

The assay was shown to be specific for the detection of Simbu serogroup orthobunyaviruses, as no cross-reactions were observed in vitro against a wide range of viral causes of ruminant abortions, such as pestivirus (BVDV-1), herpesvirus (BoHV-1), orbivirus (BTV-1 and PALV), ephemerovirus (BEFV), phlebovirus (RVFV) and flavivirus (WSLV). The latter five viruses are also arthropod-borne. In silico specificity analysis showed that sequences producing significant alignments were only from the Simbu serogroup, and no cross-reactivity was observed, especially with other genetically related viruses which may be differential diagnosis along with Simbu serogroup viruses, namely those belonging to the Bunyamwera serogroup that also can cause abortion and teratology in ruminants.

3.6.4. Repeatability

The intra-run SD ranged from 0.50 and 0.71 for probe Simbu_CladeAP and 0,14 to 0,68 for probe Simbu_CladeBP. The inter-run SD ranged from 0.19 to 0.60 for probe Simbu_CladeAP and from 0.31 to 0.97 for probe Simbu_CladeBP. CV ranged from 1.36% and 2.01% for probe Simbu_CladeAP and 0.63% and 2.91% for probe Simbu_CladeBP detection (Table 4).

3.7. Comparison with a pan-Simbu assay (Fischer et al., 2013)

The assay described by Fischer et al., (2013) was reported to detect all the tested viruses, which included AINOV, AKAV, DOUV, PEAV, SABOV, SANV, SATV, SHAV, SHUV, SIMV, THIV, TINV and SBV from clade B and OROV from clade A. This assay was compared to the novel group-specific RT-qPCR assay described in the present article. The Fischer assay detected all the tested viruses, AKAV, SIMV, SHUV, SATV, SHAV and SABOV from clade B and INGV from clade A, but the novel group-specific RT-qPCR showed 101.6 - 104.4 higher sensitivity (Table 5).

4. Discussion

We describe a novel group-specific one-step TaqMan® based RT-qPCR, targeting the S segment of the Simbu serogroup orthobunyaviruses, and designed to discriminate the two phylogenetic clades.

The design of the assay was complicated by the fact that Simbu serogroup viruses show high genetic diversity (Appendix A), as result of the lack of proofreading ability of the RdRp enzyme (Elliott, 2014) and conserved regions within the genome were very limited. The genetic diversity of the Simbu serogroup is higher relative to other orthobunyaviruses and may be as a result of the wide distribution of the group (Saeed et al., 2001), resulting in geographical isolation of viral metapopulations, which may promote divergent evolution.

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The M segment of the Simbu serogroup viruses showed a higher genetic diversity than the L or S segments. The M segment encodes the surface glycoproteins, which are major immunogens (Roman-Sosa et al., 2016; Wernike et al., 2017) and the higher diversity within the M segment may be an evolutionary result to escape immune pressure, or the necessity to infect mammal, bird and insect vectors alternately (Saeed et al., 2001; Yanase et al., 2012).

The DVTD Simbu serogroup isolates used in the development of this assay were sequenced and the prototype strain of SATV (IG_10310), SHUV strain 8912 and SABOV strain Cu 1/70 were sequenced for the first time. The number of nucleotide differences between the sequenced prototype strains and the other published sequences of the same strain, ranged from 0 to 42 nucleotides within the 699 nucleotide length of the ORF of the S segment. These polymorphisms reflect the high mutation rate of RNA viruses during viral passage in cell culture (Combe and Sanjuán, 2014; Duffy, 2018; Furio et al., 2005), which ranged from between one to five passages in our laboratory, or may have been due to sequencing errors.

The Simbu assay demonstrated good amplification efficiencies (90 - 100%) for most of the isolates, with the exception of SHAV (84%) and SABOV (110%). Efficiency values of less than 90% may be caused by Taq DNA polymerase contamination with inhibitors, inappropriate annealing temperature or poorly design primers, whereas efficiency values greater than 100% usually result from contamination with non-specific products or primer dimers (Tenreiro et al., 2014).

The Simbu assay was shown to be sensitive, ranging from 10-3.61 to 100.39 TCID50/reaction. Tauscher et al. (2017) reported the end-point sensitivity of three individual RT-qPCR assays targeting SABOV, SIMV and SATV. Up to 4.75×10−6 TCID50/ml SABOV could be detected using a SABOV-specific assay, 5.63×10−5 TCID50/ml using a SIMV-specific assay and 5.63×10−6 TCID50/ml using a SATV assay. End-point sensitivity is a measure of the value where the target is not detected by the assay and therefore not the same as the 95% LOD. The 95% LOD of our assay in TCID50/ml ranged from 1.22×10-1 (AKAV) to 1.22×103 (INGV), with a median of 1.00×100.

The intra- and inter-run variation of the Simbu assay compared favourably with other RT-qPCR assays, e.g. assays developed for the detection of Equine encephalosis virus (EEV) (Rathogwa et al., 2014) and African horse sickness virus (AHSV) (Guthrie et al. 2013). The intra-run SD of the Simbu assay ranged from 0.14 - 0.71. For the EEV assay, values ranged from 0.31 - 0.47 and for the AHSV assay, values ranged from 0 - 1.66. The inter-run SD of the Simbu assay ranged from 0.19 - 0.97. For the EEV assay, values ranged from 0.16 - 2.07 and for the AHSV assay, values ranged from 0 - 1.11.

The development of the Simbu assay was carried out using a broad selection of Simbu serogroup clade B isolates, but only a clade A African isolate (INGV) was used for testing of

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the clade A isolates. To better clarify the capacity to detect clade A isolates, further testing with other clade A isolates, e.g. OROV, is needed.

The molecular detection of a broad range of Simbu serogroup orthobunyaviruses, described by Fischer et al. (2013), used SYBR® Green chemistry for the detection of both phylogenetic clades, by targeting the L segment. Fourteen out of the 32 Simbu serogroup members were tested and was at the time of publication the first available tool for the broad screening of Simbu serogroup viruses. However, it was not specific, as it also detected Bunyamwera serogroup orthobunyaviruses. In addition, it was also not as sensitive as the assay described here, being up to 104.4 times less sensitive, depending on the virus tested (Table 5). It was not possible to replicate the exact published conditions for the assay described by Fischer et al. (2013) in our laboratory, so the conditions were not optimised in our setting and may not be sensitive as reported. Our results should be interpreted with this in mind.

Published broad-range RT-qPCR assays that target the S segment include Shirafuji et al., (2015) and Golender et al. (2018). The former is a TaqMan® assay that targets only Simbu serogroup clade B viruses, with a sensitivity reported as ten copies of AKAV, AINOV and SHAV standards, 100 copies of PEAV standard and one copy of SATV. The latter is also a TaqMan® assay, which was reported to detect 11 distinct Simbu serogroup clade B viruses, but no Simbu serogroup clade A viruses were tested. The 95% LOD of the assay was reported as 34 genome copies per reaction. A comparison between the sensitivities of these assays with the Simbu assay reported here is difficult, as the units are not comparable (genome copies and TCID50), but using a median value of 24 genome copies/TCID50 (Parker et al., 2015), the 95% LOD of the assay report by Golender et al. (2018) was calculated at an equivalent 100.15 TCID50/reaction.

We described the development of an assay with the ability to detect Simbu serogroup orthobunyaviruses, some of which have significant relevance in public and animal health. The assay was optimised and validated analytically, to provide a rapid, sensitive and specific molecular diagnostic assay. This assay may be useful in countries where Simbu serogroup viruses are endemic, where it may be used to aid in the diagnosis of clinical cases, or used for the screening of arthropod vectors or wildlife animal populations that play an important role in the epidemiology of the Simbu serogroup viruses, contributing to prevalence studies, and therefore optimising the management and control of the diseases these viruses cause. The assay may also be useful in countries where the disease is exotic and quick identification is critical, or even useful for the detection of other Simbu serogroup orthobunyaviruses that have yet to be described.

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Table 1. The Simbu serogroup orthobunyaviruses are classified into eight species and divided into two clades (ICTV). Simbu serogroup orthobunyavirus viruses used in this study are indicated in bold (DVTD, Faculty of Veterinary Sciences, University of Pretoria).

Species	Isolate	Distribution	Strain	Log TCID50/ml
CLADE A				
Oropouche orthobunyavirus	Oropouche	Central/South America		
	Facey's Paddock	Australia		
	Iquitos	South America		
	Jatobal	South America		
	Madre de Dios	South America		
	Perdões	South America		
	Pintupo	Central America		
	Utinga	South America		
	Utive	Central America		
Manzanilla orthobunyavirus	Manzanilla	Central America		
	Buttonwillow	North America		
	Oya	Asia		
	Cat Que	Asia		
	Ingwavuma	Africa, Asia	SA An 4165a,*	6.5
	Inini	South America		
	Mermet	North America		
CLADE B				
Akabane orthobunyavirus	Akabane	Asia, Africa, Australia	Ja GAr 39b	4.9
	Tinaroo	Australia		
	Sabo	Africa	AN 9398c,&, Cu 1/70d	4.3, -
	Yaba-7	Africa		
Sathuperi orthobunyavirus	Sathuperi	Asia, Africa	IG 10310e	5.3
	Douglas	Australia, New Guinea		
	Schmallenberg	Europe		
Shamonda orthobunyavirus	Shamonda	Africa	An 5550f	5.3
	Sango	Africa		
	Peaton	Australia		
Simbu orthobunyavirus	Simbu	Africa	SA Ar 53g	5.7
Shuni orthobunyavirus	Shuni	Africa	An 10107h, 8912i	4.5, -

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	Aino	Asia, Australia
	Kaikalur	Asia
Thimiri orthobunyavirus	Thimiri	Asia
UNGROUPED		
Leanyer orthobunyavirus?	Leanyer	Australia

a Isolated in South Africa from a spectacled weaver in 1959 with two passages in BHK cells.

b Isolated in Japan from pools of Aedes vexans and Culex tritaeniorhynchus mosquitoes in 1959 which, was subjected to two passages in African green monkey kidney (Vero) cells and three passages in baby hamster kidney (BHK) cells.

c Isolated in Nigeria from a goat in 1966 with two passages in Vero cells and one passage in BHK cells.

d Isolated in South Africa from Culicoides spp. in 1970 with one passage in Vero cells.

e Isolated in India from pools of Culex vishnui mosquitoes in 1957, with two passages in BHK cells.

f Isolated in Nigeria from a bovine in 1965 with one passage in Vero cells.

g Isolated in South Africa from Aedes circumluteolus mosquitoes caught during 1955 and 1957 with 2 passages in BHK cells.

h Isolated in Nigeria from a bovine in 1966, with two passages in BHK cells and two passages in Vero cells.

i Isolated in South Africa from cattle during an outbreak of a formerly unrecognised disease in 1967 with one passage in BHK cells.

* Now classified as Manzanilla orthobunyavirus (ICTV).

& Now classified as Akabane orthobunyavirus (ICTV).

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Table 2. Primers used for sequencing the S segment of the Simbu serogroup orthobunyaviruses.

Name	*Sequence (5' → 3')*	*Location*	*Length*	*Tm (°C)*	*GC%*
Akabane_AB289319F	GAACTCCACTATTAACTACGCATTG	7-32	25	62	40
Akabane_AB289319R	AAAGGTGTGCACCACATAGA	785-805	20	62	45
Simbu_NC018477F	AATGGCAAACCAATTCA	24-41	17	55	35
Simbu_NC018477R	GGCGTACAACACATAGA	787-804	17	56	47
Shuni_KU937313F	AGTGTAYTCCACTATAGAACAAAGC	5-30	25	62	40
Shuni_KU937313R	AGTGTGCTCCACATAGAACAAT	828-850	22	62	41
Sathuperi_HE795104F	CACTACTGAAATATGTCAAGCCAATTC	32-59	27	63	37
Sathuperi_HE795104R	CTCAACAGAAGCCTTGCAGTAT	805-827	22	63	46
Shamonda_NC018464F	CCACTATTAACTACAGAAATATGTCAAGCC	11-41	30	64	37
Shamonda_NC018464R	GGACCCGAAAGATGGTGAACTA	848-870	22	64	50
Ingwavuma_KF697141F	AGTAGTGTACTCCACWATTCAA	1-23	22	59	36
Ingwavuma_KF697141R	GTAGTGTGCTCCCAATTCA	955-974	19	59	48
Sabo_AF362396F	GTGTACTCCACTATTAACTACGTACC	5-31	26	62	42
Sabo_AF362396R	GAATTGGCGTGTCTCACATAGA	791-813	22	62	45

Table 3. Group-specific RT-qPCR primers and probes targeting the S segment of the Simbu serogroup orthobunyaviruses. * According to the open reading frame of NC018477.

Name	Sequence (5'→ 3')	Location*	Length	Tm (°C)	GC%
Simbu_F	TAGAGTCTTCTTCCTCAAYCAGAAGA	120-145	26	57	40
Simbu_R	TAYTGGGGAAAATGGTTATTAACCA	218-242	25	59	34
Simbu_CladeAP	VIC® -TACGTMAGACGYCGAGG-MGB	161-177	17	67	59
Simbu_CladeBP	FAM™-TYGGTTGTGSCGTCTT-MGB	166-181	16	69	53

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Table 4. Intra and inter-run variation of a group-specific RT-qPCR assay targeting the Simbu serogroup orthobunyaviruses using probe a) Simbu_CladeAP for INGV detection and b) Simbu_CladeBP for SATV detection.

a)

LogTCID50/rxn	Cq Total Mean	Cq Intra-run SD	Cq Inter-run SD	Cq Total SD	CV (%)
0.80	34.56	0.56	0.35	0.67	1.93
0.50	36.57	0.50	0.19	0.50	1.36
0.20	37.68	0.56	0.59	0.75	2.00
-0.10	38.50	0.71	0.60	0.77	2.01
-0.40	39.03	0.59	0.55	0.62	1.59

b)

LogTCID50/rxn	Cq Total Mean	Cq Intra-run SD	Cq Inter-run SD	Cq Total SD	CV (%)
-1.00	33.86	0.31	0.68	0.67	1.99
-1.30	34.88	0.38	0.49	0.57	1.64
-1.60	36.45	0.45	0.47	0.58	1.59
-1.90	37.65	0.68	0.97	1.10	2.91
-2.20	38.59	0.51	0.36	0.62	1.62
-2.50	39.50	0.14	0.31	0.25	0.63

TCID, tissue culture infectious dose; Rxn, reaction; Cq, quantification cycle; SD, standard deviation; CV, coefficient of variation.

Table 5. Quantification cycle (Cq) values obtained after testing a 1:10 dilution of DVTD virus passages with a novel group-specific RT-qPCR (this study) and a pan-Simbu assay (Fischer et al., 2013). *An efficiency of 100% was assumed.

Virus	Cq group-specific	Cq pan-Simbu	Cq difference	Fold difference*
AKAV 10-1	17.61	32.13	14.52	23496
SIMV 10-1	16.49	27.76	11.27	2465
SHUV 10-1	19.14	33.37	14.23	19175
SATV 10-1	27.91	33.75	5.84	57
SHAV 10-1	21.52	28.75	7.23	150
INGV 10-1	22.46	36.91	14.45	22431
SABOV 10-1	26.79	32.21	5.41	43

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Fig. 1.

```
            120       130       140       150       160       170       180       190       200       210       220       230       240
            |....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|..
AB426280 AKAV 37  tagagtcttcttcctcaaccagaagaaggccaagatggtcttacataagacgccacaaccaagtgtcgatcttacttttgcaggggtcaaatttacagtggttaataaccattttccccagta
AB373234 AKAV 2   ...........................................................................................................................a..
AB000819 TINV     ..............................................................................................................................
AF362396 SABOV 2  .c...................a..a........a..................g................c..........gt...........c..........................a..
HE795092 DOUV 2   ..........................................g...........................gt...........c..........................a..
HE795104 SATV 2   ...................................c..........g...........................gt...........c..........................a..
AB698468 SATV 3   ...................................c..........g...........................gt...........c..........................a..
AB698469 SATV 2   ....................a..................c..........g...........................gt...........c..........................a..
NC018464 SHAV 9   ..............................a......c..........g...........................gt...........c..........................a..
AF362404 SHAV     ..a..............................a......c..........gg..........................gt...........c..........................a..
LC309141 SBV 6    ......................................c..........g...........................gt.......................................a..
LC309142 SBV      ......................................c..........g............c..............gt.......................................a..
LC309144 SBV      ......................................c..........g..................c.......gt.......................................a..
AF362392 Y7V      ..........................a...........c..................g..............a.....t...........c...........................
NC018477 SIMV 3   ................................a.................g.........a...t............g...............c..........a.........a..
KU937313 SHUV 17  ......................................ac.cag.......g.......g.....t........a....gt..ca...........c.....................a..
KT946779 SHUV 2   ......................................ac.cag.......g.......g.....t........a...agt..ca...........c.....................a..
HE800143 SHUV     .................t...................ac.cag.......g.......g.....t........a....gt..ca...........c.....................a..
AF362405 SHUV     .................t.....................c.cag.......g.......g.....t........a....gt..ca...........c.....................a..
KC510272 SHUV     .................t...................ac.cag.......g.......g.....t........a....gt..ca...........t.....................a..
AF362394 KAIV     .................t...................ac.cag.......g.............t........a....gt..ca...........c.....................a..
AB334170 AIVOV 5  .................t....................gc.cag.......g.............t........a....gt..ca...........c.....................a..
AB334188 AIVOV 4  .................t....................gc.cag.......g.............t........a....gt..ca...........t.....................a..
NC018460 AIVOV    .................t....................ac.cag.......g.............t........a....gt..ca....g......c.....................a..
M22011   AIVOV    ....t............t...................ac.cag.......g.............t........a....gt..ca....g......c.....................a..
AB542965 PEAV 5   .................t.....................c.cag.......g.......g.....t........a....gt..ca...........t.....................a..
AY048678 PEAV 2   .................t....................gc.cag.......g.............t........a....gt..ca...........t.....................a..
AB542964 PEAV     .................t.....................c.cag.......g.............t........a....gt..ca...........t.....................a..
AF362402 SANV 2   .................t....................gc.cag.......g.......g.....t........a....g...ca...........t.....................a..
JQ029991 INGV 3   .c....t...........t.......ga.....agatactc...g.c.......gagg..c.a...ta............g.ca.tg..c....c......................a..a.t
KF697141 INGV 2   .c..a.t...........t.......ga......gatact....g.c.......gagg..c.c....a.............g.ca.tg..c....c.................a...a..a.t
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KP691605 OROV 10  ....................a...........agat.......g......at.gagg..c.tg..t..c..........gt......c.....g..a................c..a....t
KP691629 PDEV     ....................a...........agat.......g......at.gagg..c.tg..t..c..........gt......c.....g..a................c..a....t
KJ866386 IQTV 15  ....................a...........agat.......g......at.g.gg..c.tg..t..c..........gt......c.....g..a................c..a..a.t
KF697144 IQTV     ....................a...........agat.......g......at.gagg..c.tg..t..c..........gt......c.....g..a................c..a..a.t
AF312382 JATV     ....................a...........agat.......g......at.gagg..c.tg..t..c..........gt......c.....g..a................c..a..a.t
JQ675601 JATV     ....................a............gat.......g.......t.g.gg..c.tg..t..a..........g......t.........a................c..a..a.t
KF697148 MANV     ....a.a...........t.......ga.....agatact....g.c.......gagg..c.c....a............g.ca.tgt.c....c..................c..a..a.t
KP016014 MANV     .c....t...........t.......ga.....agatact....g.c.......gagg..c.a...ta............g.ca.tg..c....c.....................a..a.t
KJ866389 MDDV 2   ....................a..a........agat.......g.......t.gagg..c.tg..t..c..........gt.....gc.....g..a................c..a..a.t
NC024075 CQV      .c....t...........t.......ga.....agatact....g.c.......gagg..c.a...ta............g.ca.tg..c....c.....................a..a.t
JX983192 OYAV     .c....t...........t.......ga.....agatact....g.c.......gagg..c.a...ta............g.ca.tg..c....c.....................a..a.t
KY795950 OYAV     .c....t...........t.......ga.....agatactc...g.c.......gagg..c.a...ta............g.ca.tg..c....c.....................a..a.t
KF697152 MERV 3   .c..a.t...........t.......ga......gatact....g.c.......gagg..t.c....a............g.ca.tgt.c....c.....................a....t
KF697136 FPV 2    .c..a.t..........................gatcgtc.tgc.......g.gag.g...c...t...a....g....g.......gtc...c..........c.......a...ag.a..
KF697162 BUTV 2   ....a.t..........................gatcgtc...g.c.....t.........a....a............gcca.tcagtc...c.....................a..a.t
KF697156 UTIV     ......g.....tt.a.....a.....t..t..a.at..t..gaga..a..ct..ag.....a...g..ct.g..a....gg..t.ca.cc...c..a.t..c..c...........t..a.t
KF697158 UVV      ......g.....tt.a.....a.....t..t..a.at..t..gaga..a..ct..ag.....a...g...t.g..a....gg..t.ca.cc...c..a.t..c..c...........t..a.t
                  tagagtcttcttcctcaaYcagaaga              ttctgcSgtgttggYt                              accaattattggtaaaggggtYat
                                             tacgtMagacgYcgagg
```

Fig 1. Nucleotide variation within the region of the S segment (nucleotides 120-242, using the open reading frame of NC018477 for numbering) of Simbu serogroup orthobunyaviruses (n = 167), targeted by a novel group-specific RT-qPCR assay. The primer and probe sequences of this assay are indicated at the bottom of the figure. Number suffix in the sequence title indicate the number of identical Simbu serogroup sequences to that sequence. Dots in the sequence indicate identity with the first sequence. Primers are indicated by the grey blocks, probe Simbu_CladeBP is indicated by the blue block (for Akabane, Tinaroo, Sabo, Douglas, Sathuperi, Shamonda, Schmallenberg, Yaba-7, Simbu, Shuni, Kaikalur, Aino, Peaton and Sango virus detection) and probe Simbu_CladeAP is indicated by the green block (for Ingwavuma, Oropouche, Perdões, Iquitos, Jatobal, Manzanilla, Madre de Dios, Cat Que, Oya, Mermet, Facey's Paddock, Buttonwillow, Utinga and Utive virus detection).

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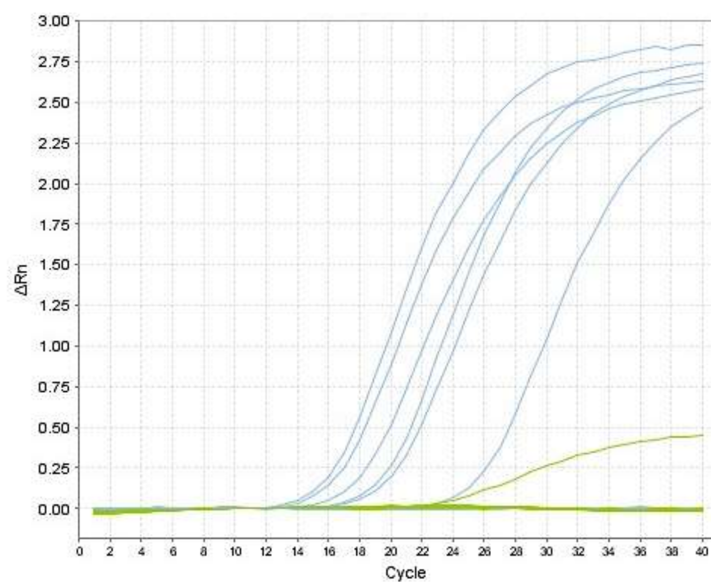

Fig 2. Amplification curves of the group-speciifc real-time RT-PCR plotted as fluorescence intensity (Rn) against cycle number showing successful amplification of AKAV, SIMV, SHUV, SATV, SHAV, SABOV (blue curves), and INGV (green curve). Negative control is represented by the flat line.

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Fig 3

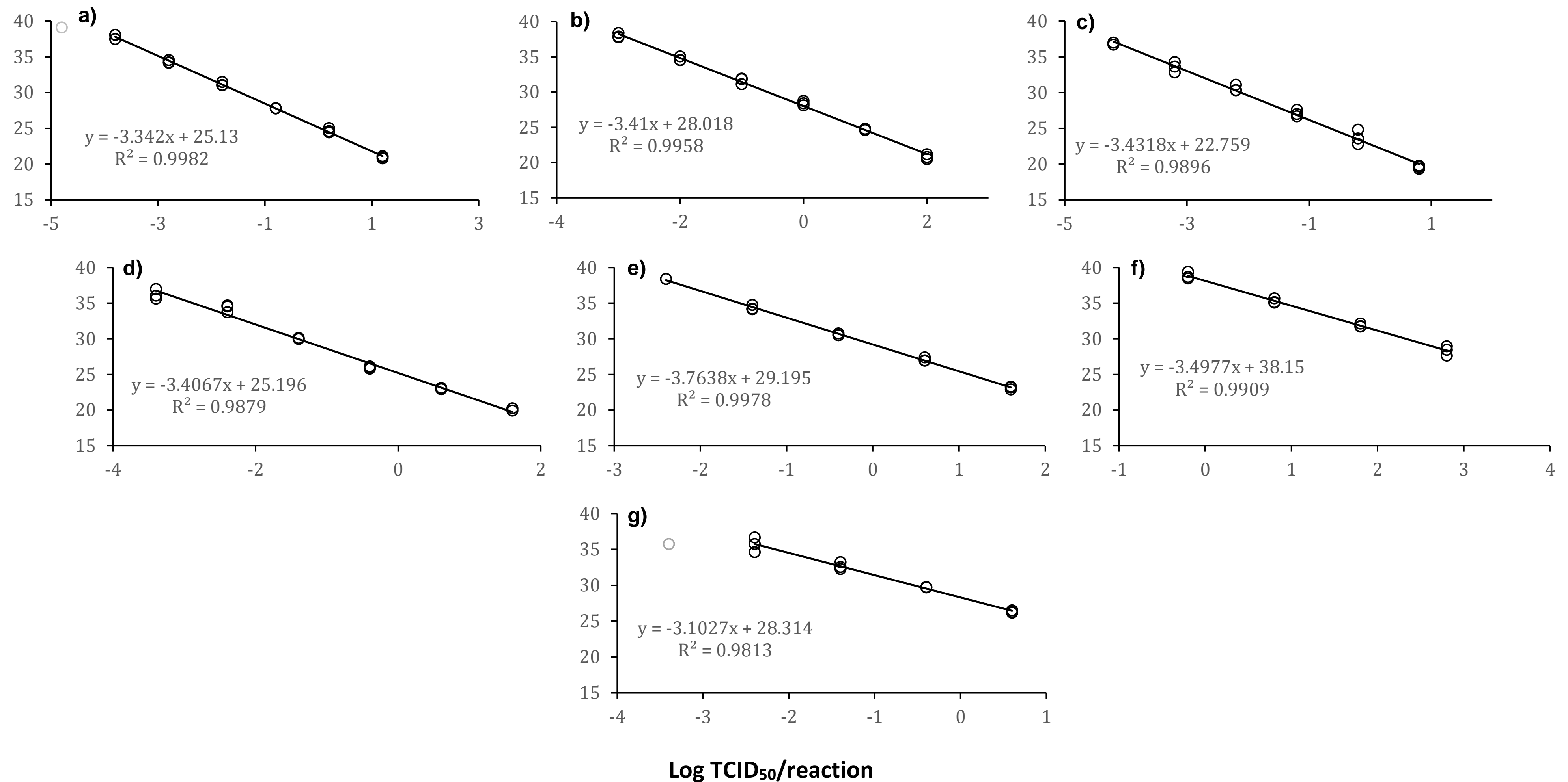

Fig 3. Efficiencies of a group-specific Simbu Orthobunyavirus assay, determined by regression analysis of a ten-fold dilution series of tissue cultured AKAV (a), SIMV (b), SHUV (c), SATV (d), SHAV (e), INGV (f) and SABOV (g). Each dilution had three replicates (black circles). Outliers were not included in the calculation (grey circles).

Appendix

Fig. A1 DVTD sequence alignment.

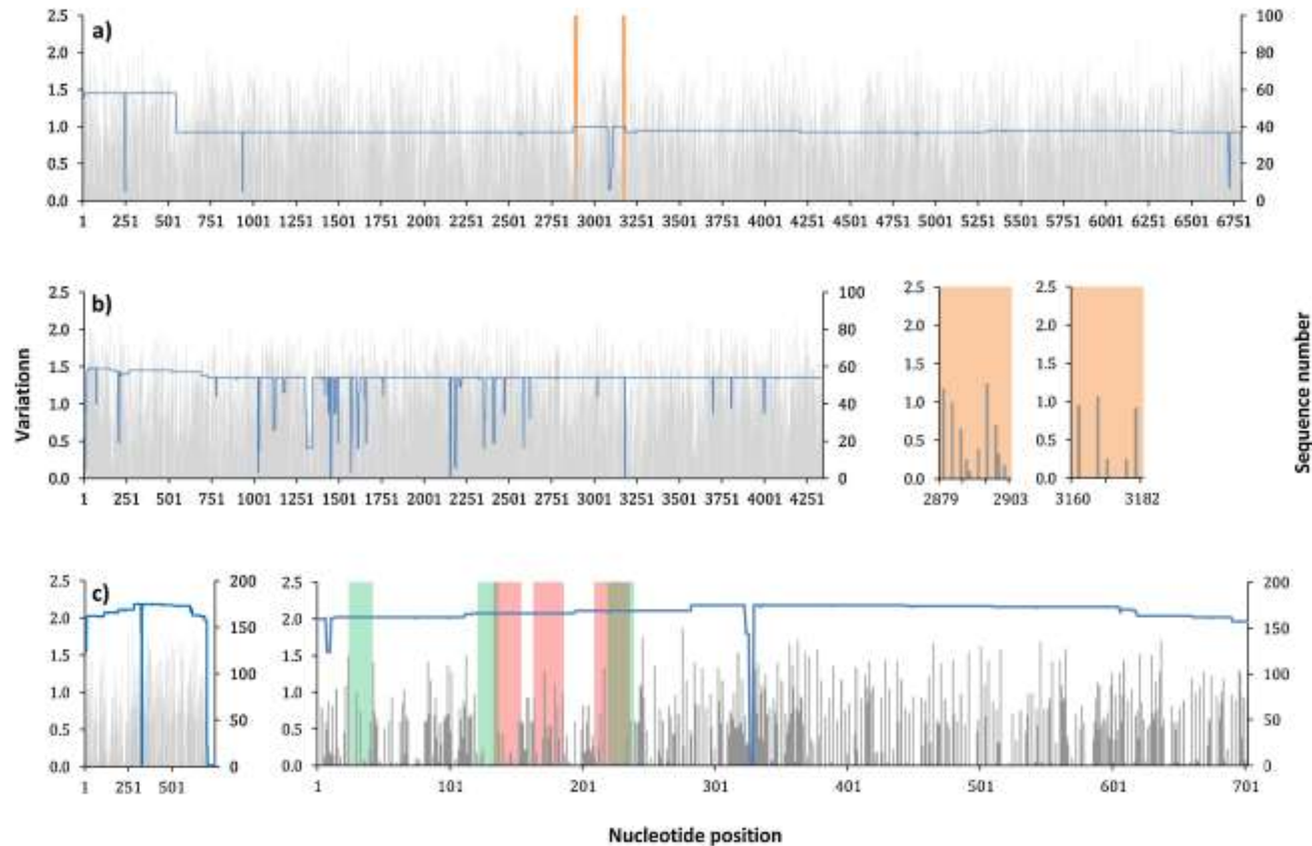

Fig. A1. The high nucleotide variation of the Simbu serogroup orthobunyaviruses is shown for the L (a), M (b) and S (c) segments, showing very few conserved regions. The S segment is shown twice, first in a view relative to the other segments (M and L), and then in an expanded view to better illustrate conserved regions. Column height indicates the variation in each nucleotide position: the higher the column, the greater is the variation. All relevant sequences available in Genbank® were used, and the number of sequences analysed at each nucleotide position is indicated by the blue line. Coloured columns indicate the position of primers/probes of published group-specific RT-qPCR assays: orange (Fischer et al., 2013), red (Shirafuji et al., 2015) and green (Golender et al. 2018).