

A Sensitive Nested Real-Time RT-PCR for the Detection of Shuni Virus

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SUMMARY

Recently Shuni virus (SHUV) has been identified in clinical cases of neurological disease in horses in South Africa. Being that it was one of the less recognized orthobunyaviruses, with limited clinical descriptions of disease dating back to the 1960s and 1970s, SHUV-specific assays were never developed. In this study we report the development of a nested real-time PCR assay for the detection of SHUV by means of melt-curve analysis using fluorescence resonance energy transfer (FRET) probe technology. The assay was validated against previously positive clinical specimens and a dilution series of controls. This assay was applied in the screening of 386 clinical specimens of horses with neurological signs. SHUV could be amplified from cell culture, blood and tissue specimens from horses with acute disease. This is a rapid and sensitive assay that may be implemented in both diagnostic and research laboratories.

Keywords

Shuni virus, Real-time RT-PCR, FRET probes, diagnostic PCR

1. Introduction

Shuni virus (SHUV) was first isolated in 1966 from a cow in Sokoto, Nigeria during a surveillance program for the study of viral infections that was instituted by the University of Ibadan between 1964 and 1969. During the course of the survey SHUV was also isolated twice again from cattle and once from a sheep and neutralizing antibodies being found in dairy, trade cattle as well as sheep (Causey et al., 1972; Causey et al., 1969; Kemp et al., 1973). SHUV was also twice isolated from *Culicoides ssp.* during field surveillance (Lee, 1979). A single human isolation of SHUV was reported from a one and a half year old child at the General Outpatients Clinic at the University College Hospital (UCHGOP) in August 1966, as part of the arbovirus surveillance initiative in Nigeria (Moore et al., 1975).

In South Africa, SHUV was recovered twice from pools of *Culex theileri* mosquitoes caught near Johannesburg and from 7 apparently healthy cattle and a goat in Natal between the years 1970 and 1979 (McIntosh, 1980; McIntosh et al., 1972). In 1977, the virus was isolated from the brains of two horses with nervous disease submitted for rabies virus examination; one from South Africa (Coetzer and Howell, 1998) and one from Zimbabwe (Coetzer and Erasmus, 1994). Considering the public and veterinary health importance of other members of the *Orthobunyavirus* genus, surprisingly little attention was placed on uncovering the role SHUV may play in encephalitic disease in humans and animals. The identification of SHUV in 7 horses with neurological symptoms between June 2009 and December 2010 (van Eeden et al., 2012), led us to believe that this virus may play a noteworthy role in neurological disease in South Africa. To enable the rapid identification of SHUV in acute cases submitted through the zoonotic arbovirus sentinel surveillance programme for neurological disease in animals, and to enable other laboratories to search for this virus in their region, the aim was to develop a sensitive and specific molecular diagnostic test for acute cases.

A nested PCR for the detection of Shuni virus which was previously described (van Eeden et al., 2012), although effective is less practical in a diagnostic setting due to the need to confirm diagnosis by nucleic acid sequencing of amplicons. To improve on the turnaround time, sensitivity and specificity of this test we sought to design a real-time assay to incorporate into a diagnostic setting. This assay can be implemented in both diagnostic and research laboratories, allowing for further investigations into the prevalence and association of SHUV with acute neurological disease in animals and humans in Africa as well as in other continents. In light of other emerging orthobunyaviruses; Schmallenberg virus in Europe

(Hoffmann et al., 2012) and Iquitos virus in Peru (Aguilar et al., 2011) which have been described recently, the investigation of Shuni virus as a cause of unexplained outbreaks of neurological disease, especially in horses is warranted.

This report describes the development of a sensitive asymmetric nested real-time PCR assay, incorporating fluorescence resonance energy transfer (FRET) probes for the rapid detection of SHUV in diagnostic, surveillance and epidemiological studies. The assay was assessed for sensitivity and specificity against a dilution series of a cloned first round PCR product of known concentration. The assay was validated against known positive cases of Shuni virus and was applied in the screening of 386 unsolved cases of neurological disease in animals in South Africa.

2. Methods

2.1 Virus strains

Three SHUV strains from horses which had displayed neurological symptoms and had proved to be positive for SHUV through nested PCR (van Eeden et al., 2012), were used for the optimization of the real-time assay, these included a cell culture isolate (SAE 18/09), a brain specimen (SAE 72/09) and a blood specimen (SAE 27/10)..

2.2 Preparation of positive control for quantification

A first round PCR product from the cell culture isolate SAE 18/09 was cloned and the concentration of the amplicon determined through use of the NanoDrop 3300 (Thermo Scientific, Wilmington, USA). A dilution series of this was then used to determine the detection limit of the assay through calculation of copy number.

2.3 Primer and Probe design

The SHUV-specific primers previously described (van Eeden et al., 2012); SHUVS111+ and SHUV688- (Table 1) were used in combination with Fluorescence Resonance Energy Transfer (FRET) probes that were designed with the aid of the LightCycler Probe design software package (Roche Applied Science, Mannheim, Germany): SHUV Probe 1 and SHUV Probe 2 (Table 1). A highly conserved region of the N protein was selected relative to the prototype SHUV strain and other members of the Simbu serogroup.

Table 1. Primer and probe sequences for nested real-time PCR

Primer	Orientation	Sequence	Genome position (bp) ^a	Reference
SHUVS111+	Sense	CGA TAC CGT TAG AGT CTT CTT CC	111-134 (S)	(van Eeden et al., 2012)
SHUVS688-	Anti-sense	CGA ATT GGG CAA GGA AAG T	688-706 (S) rc	(van Eeden et al., 2012)
SHUVS178+	Sense	CCG AGT GTT GAT CTT ACA TTT GGT	178-202 (S)	(van Eeden et al., 2012)
SHUVS611-	Anti-sense	GCT GCA CGG ACA GCA TCT A	611-630 (S) rc	(van Eeden et al., 2012)
Probe	Orientation	Sequence	Genome position (bp) ^a	Reference
SHUV Probe 1	Sense	ATA TGC ACA GAG TGC TAA AAG ATG GGA TG -P ^b	488-516 (S)	This study
SHUV Probe 2	Sense	LC red 640 - GTC AAC TTC ATG CGA AAG GTT CTT CGC CAA CG -P ^c	520-551 (S)	This study

^a SAE18/09-KCS10272
^b F = Fluorescein
^c P = Phosphate

2.4 RNA extraction

RNA was extracted from cell culture or EDTA blood using the QIA-amp viral RNA mini kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. For fresh tissue samples, approximately 30mg pieces were extracted with the RNeasy Plus mini kit (Qiagen, Valencia, CA). The same methods were employed for the extraction of the 386 retrospective specimens.

2.5 Reverse transcription PCR

RT-PCRs were performed using the Titan One Tube RT-PCR Kit (Roche, Mannheim, Germany) and SHUV specific primers SHUVS111+ and SHUVS688-. Briefly, 10µl RNA was added to 10µl 5x reaction buffer, 5mM DTT solution, 40pmol of each primer, 10mM of each dNTP, 1µl Titan™ enzyme mix, 10 U of Protector RNase inhibitor (Roche, Mannheim, Germany) to a final volume of 50µl. The reaction mix was subject to initial incubations of 50°C for 40 minutes, 94°C for 2 minutes followed by 35 PCR cycles: 94°C, 30 sec; 52°C, 1 min; 68°C, 1 min, supplemented by a final extension for 7 min at 68°C

2.6 Real-time PCR with FRET Probes

A nested real-time PCR was carried out on the first round products using the FastStart DNA Master Plus Hybprobe kit (Roche Applied Science, Mannheim, Germany) according to the manufacturers' recommendations and performed on a LightCycler 2.0. Each reaction contained 2µl of the first round PCR product, 10 pmol of each probe (Probe 1 and Probe 2), 5 pmol of SHUVS178+, 25 pmol of SHUVS611 (Table 1) (Concentrations of 5 to 50 pmol of SHUVS611 were assessed during the optimisation process) and 4µl of enzyme master mix in a 20µl reaction. Reactions were cycled as follows: 95°C for 10 min, followed by 45 cycles of 95°C for 10 sec, 50°C for 8 sec, 72°C for 8 sec, followed by melting curve analysis between

30 and 80°C, at a temperature ramp rate of 0.1°C/s. A product of 430bp could be visualized on an agarose gel and be used for sequencing.

2.7 Sequencing

Amplicons were sequenced using the Big Dye[®] Terminator V3.1 Kit as recommended by the supplier (Applied Biosystems, Foster city, CA). Reactions were precipitation using the EDTA/NaOAc/EtOH method according to the BigDye Terminator v3.1 cycle sequencing protocol (Applied Biosystems, 2002). Sequences were edited using Sequencher v4.6 and confirmed to be SHUV by BLAST search analysis. Sequences were aligned using the ClustalW subroutine, which forms part of the Bioedit program (Hall, 1999). P-distance analyses were carried out for nucleotide sequences using Mega v5 (Tamura et al., 2011).

3. Results

3.1 Real-time PCR development and optimisation

A nested real-time PCR with SHUV specific FRET probes was developed for direct detection of SHUV by melting curve analysis in clinical specimens. Two SHUV strains, a cell culture isolate and a brain specimen were used to test the efficiency of the assay. Both specimens could be detected with the SHUV nested real-time assay after melt curve analysis (Fig. 1), however a double peak with melting temperatures of 59°C and 65°C was observed when using symmetric primer concentrations, although only a single band was visible by agarose gel electrophoresis (Fig. 1). Neither sample concentration nor annealing temperature affected this outcome. To optimise the melting curve analysis, an asymmetric PCR was used where the concentration ratios between the forward and reverse primers are adjusted. It was found that at a forward to reverse primer ratios of 1:5 to 1.6, the signal intensity increased and the additional peak disappeared (Fig. 1). It was with these parameters that the assay was further evaluated. Computational analysis was carried out to assess the specificity of the assay, as no other Simbu serogroup viruses were available for experimental evaluation. Results showed that the mismatches between the different viruses are significant enough to type the viruses correctly (Fig. 2), due to these mismatches the predicted T_m could only be predicted for SHUVs closest related virus (55.5°C). Based on the similarity of the predicted (64.1°C) and experimental (64.7-65.7°C) values obtained for SHUV the experimental values for the other Simbu serogroup members are likely to closely reflect the predicted values.

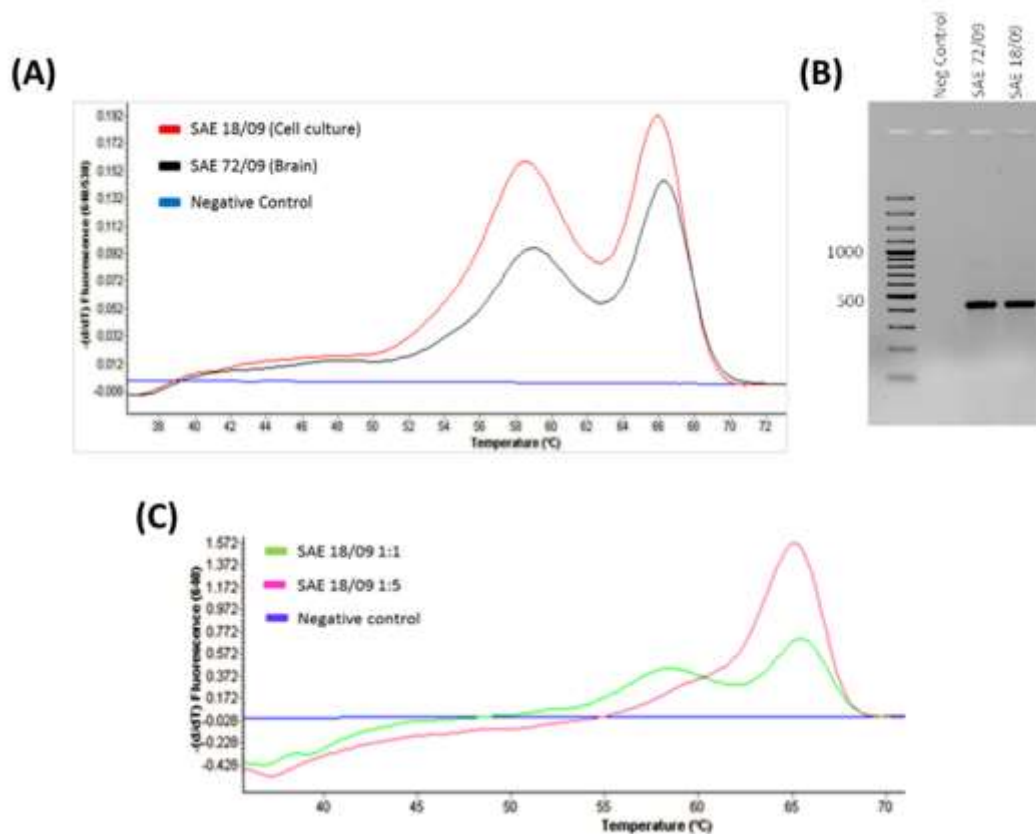


Fig. 1. Nested real-time PCR assay optimization. **(A)** Melting peaks showing both the cell culture isolate SAE 18/09 and the clinical specimen SAE 72/09 positive for SHUV following nested real-time PCR with a 1:1 primer ratio. **(B)** Agarose gel showing real-time PCR products. **(C)** Melting peaks showing the detection of SAE 18/09 at both a 1:1 and 1:5 forward/reverse primer ratio. A ratio of 1:1 produced 2 peaks (59°C and 65°C) and a ratio of 1:5 only one (65°C).

Probes	ATATGCCACAGAGTGCTAAAAGATGGGATGG---TCAACTTCATCGGAAAGGTTCTTCGCCAACG	Predicted T _m	Experimental T _m
SHUV (KC510272)	.C.....ATG.....	64.1 °C	64.7/65.7°C
SHUV (HE800143)	.C.....ATG.....	64.1°C	NA
AINOV (HE795089)G.G...A..ATG.....A.G...C...G..	55.5°C	NA
PEAV (HE795095)	.C...T...A...A...ATG...T..A...A..C.....	*	NB
KALV (AF362394)	.C.....G..G..C...ATG...T..A.G...C.....G..	*	NB
SINV (HE795110)T...TT.....C...ATG.A...T...G..A.C...GA.	*	NB
BUTV (AF362398)	.A.....AC.C.....A...ATG.....AAG..A..AA.AA.G..GA.	*	NB
SABOV (HE795098)TT.G..G.....ATG.....G..A..A..C...G..	*	NB
TINV (AB000819)	.C.....C.T..A.....ATG.....A.G...C..A...GA.	*	NB
AKAV (AB000851)	.C.....C.T..A...G...A...ATG.....A.G...CT.A..T..GA.	*	NB
DOUV (HE795092)	.G.....T...C..C...AAT...ATG.G.....AAG..A..C.....	*	NB
FPV (AF362400)	.A.....G...CT.G..G...A...ATG.A...T...C..A..AT.G...A.	*	NB
INGV (AF362395)	.G.....C.T..TT.G.....C...AAG..A..T...A.G...G.....	*	NB
MERV (AF362399)	.G.....C.T...C...C...ATG.A.....G.....C...G..	*	NB
SANV (HE795101)	.C.....T...G..C..A...ATG.....T..A...A..C..C..T..G..	*	NB
SATV (HE795104)	.G.....G..T..C...AAT...ATG.A..T...AA...A..C.....G..	*	NB
SHAV (HE795107)	.A.....T..G..C..T..G..CAAT...ATG.G..T..T...AA...A..C..C.....	*	NB
OROV (AF164549)	.G.....G..C..C..G..C..C...ATG.....T...AAG..A..C...G..GA.	*	NB
SBV (HE649914)	.A.....T..G..C..C..G..CAAT...ATG.A..T..T...AA...A..C..C.....	*	NB
Y7V (AF362392)	.C.....TC.C..CT.....C..A...ATG.G.....A...CT.A...GA.	*	NB

* Contains too many mismatches for melting temperature prediction
 NA Not available for testing
 NB Probes do not bind

Fig. 2. Comparison of FRET probe sequences to other members of the Simbu serogroup. Dots indicate conserved bases, whereas sequence variations are indicated by nucleotide bases. Probe melting temperatures that could be predicted or experimentally determined are also indicated.

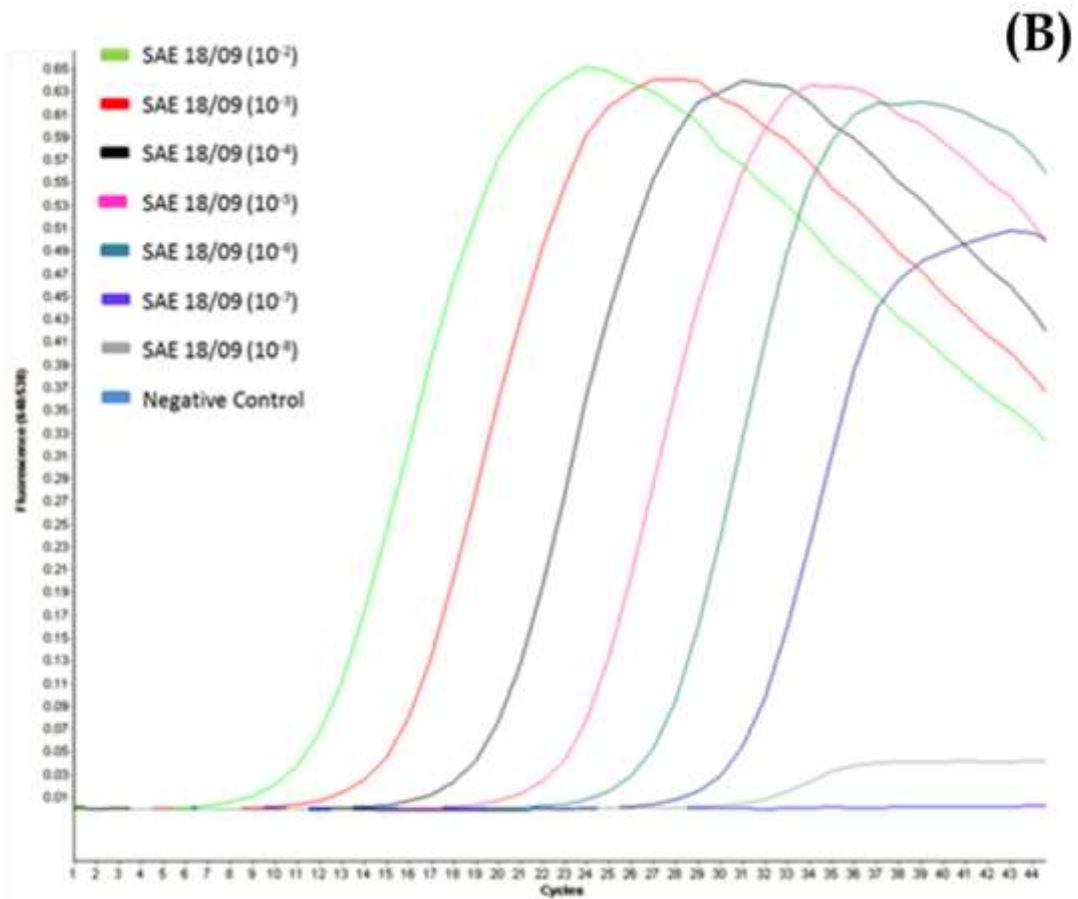
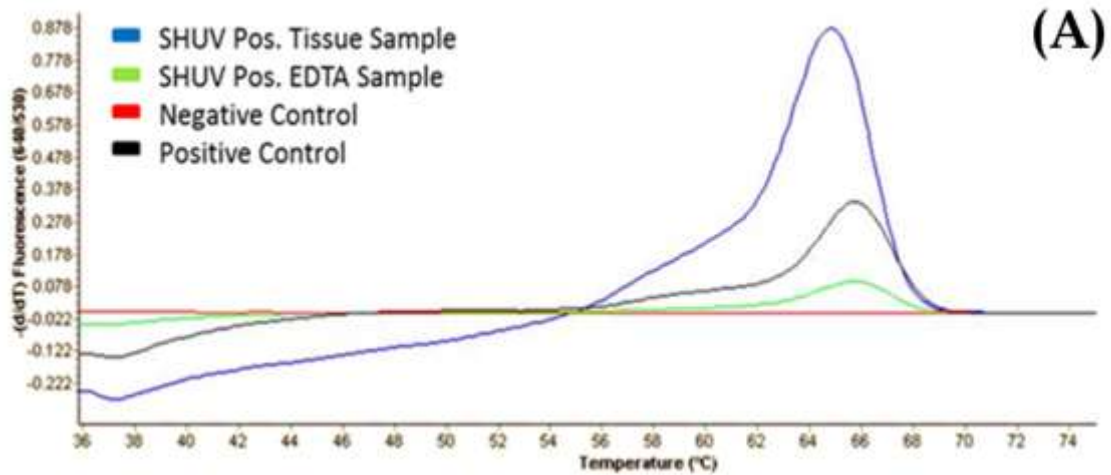


Fig. 3. (A) Melting peaks showing two clinical specimens positive for SHUV following nested real-time PCR. The specimens included a brain sample from a horse that had succumbed to neurological disease and blood from another horse which had survived the infection. **(B)** Amplification curve analysis of a dilution series of SAE 18/09 cloned RNA control, indicating the sensitivity of the nested real-time assay.

3.2 Evaluation against known positive specimens

Clinical specimens in the form of brain from a horse that succumbed to neurological disease and blood from another horse which survived were, used to test the diagnostic ability of the assay. SHUV was amplified directly from RNA extracted from the two clinical specimens (Fig. 3), where detection signals became apparent after 10 cycles of the nested real-time PCR. These results could be confirmed with the conventional nested PCR (van Eeden et al., 2012). The specimens exhibited melting peaks of between 64.76°C and 65.73°C, which correlated well with the 65.78°C peak observed for the positive control. The sensitivity of the assay was determined by performing the PCR on a dilution series of cloned virus (SAE 18/09) for which the plasmid copy number had been calculated (Fig. 3). The detection limit was determined to be 1.79×10^{-3} viral genome copies/ μ l as previously described. The assay was then further validated by running clinical samples that were shown previously to be positive, in triplicate.

3.3 Evaluation against unsolved cases of neurological disease in horses

An additional 386 blood and other tissue samples from horses submitted between 2008 and 2012 which had displayed neurological signs were retrospectively screened.. Two additional SHUV cases, both from blood samples of horses with neurological symptoms were identified. Both cases occurred in 2011, the first in February from a horse from the Mpumalanga province (SAE 15/11) that succumbed following neurological signs, the second was submitted in April from a horse with neurological signs from the Northern Cape Province (SAE 87/11) that survived with no significant sequelae. This brings the total number of SHUV cases detected in the specimens from horses with neurological signs to 9 (van Eeden et al., 2012). Detailed epidemiology will be reported elsewhere.

3.4 Sequence confirmation of newly identified cases

All cases identified by the nested real-time PCR were confirmed by sequencing, blast and phylogenetic analysis of the amplicons. The additional two SHUV amplicons were sequenced and the data deposited in GenBank (accession numbers): SAE 15/11 – KC525996, SAE 87/11 – KC525997. Analysis of the genetic distances between the amplified region of the N-protein of the SHUV strains, showed the intrinsic variation between them to be low (0.9-2.2%) and that the 9 SHUV strains averaged a 4.3% difference to the prototype SHUV isolate.

4. Discussion

In this study an asymmetric nested real-time PCR is described for the rapid identification of Shuni virus, an assay which may have applications in diagnosis as well as epidemiology and surveillance programs. This is particularly relevant following the identification of SHUV as a possible cause of neurological disease in horses in South Africa (van Eeden et al., 2012) and the previous identification of this virus in Zimbabwe and Nigeria which suggests it may occur across the African continent. A sensitive real-time assay will facilitate the diagnosis of this virus in animals with neurological signs and epidemiological investigations in different geographic areas.

The S segment of the genome was selected as the target for this assay due to the high level of conservancy of this segment. The M segment of the orthobunyaviruses in particular is prone to reassortment and variants have been identified in members of the Simbu serogroup, thus making this segment an unsuitable target for diagnostic and surveillance programs. For evolutionary studies however the more variable M and L segments would be more appropriate. The nested real-time PCR approach greatly enhances the sensitivity of this assay, which may increase the time period in which viral RNA can be detected in clinical disease before the clearance of viremia. This increased sensitivity will also have a significant impact on surveillance studies, where low concentrations of virus need to be detected in pooled specimens. Although the risk of contamination is increased with the nested PCR approach incorporation of Uracil-DNA-Glycosylase (UNG, Roche Applied Science, Mannheim, Germany), may prevent this. Alternatively the assay may be run as a single round PCR using primers SHUV178+ and SHUV611-, following cDNA synthesis with random hexamer primers, this proved efficient for the amplification and detection of the control SAE 18/09, where a comparable detection limit was observed (Results not shown).

FRET probes combine the advantages of real-time with the ability to type samples, where sequence variation between the probe and template can be exploited by melting curve analysis thus eliminating the need for time consuming sequence and phylogenetic analysis. The specific melting temperature for a probe-template interaction will be determined by the number and location of mismatches between them, thereby generating distinct melting curves for different mismatch patterns (Yeh et al., 2004). Analysis of the Simbu serogroup viruses showed sequence variations at the probe binding site should be suitable to distinguish between viruses based on the predicted melting temperatures, these viruses could however not

be obtained for experimental analysis. The high sequence diversity observed between the probes and other orthobunyaviruses suggests that these will not be detected by the SHUV probes.

Initial experiments with symmetric primer concentrations resulted in a bi-modal peak, following melt curve analysis. Changes in virus concentration and annealing temperature did little to rectify this and thus an asymmetric PCR (AS-PCR) strategy was considered. The significance of AS-PCR in dual probe systems has been highlighted in many studies where it has been shown that competition between the complementary strand of DNA and the probes, as well as incorrect genotyping are reduced (Bernard et al., 1999; Burggraf et al., 2002) and that fluorescent intensity and specificity in melt curve analysis is significantly increased (Szilvasi et al., 2005).

In this study it was found that at a forward to reverse primer ratio of 1:5, the secondary peak previously seen, disappeared. The sensitivity of the assay was demonstrated by amplification in both a tissue (brain) and a blood sample, both samples were from horses that had displayed neurological symptoms and had been confirmed previously as SHUV positive by conventional nested PCR (van Eeden et al., 2012) and sequencing. The experimental melting temperatures of 64.7°C and 65.7°C correlated well with the predicted melting temperatures as calculated by the LightCycler Probe design software package (Roche Applied Science, Mannheim, Germany). The real-time PCR products could be visualized by agarose gel electrophoresis and used for nucleotide sequencing.

The assay was then applied to screen an additional 386 specimens collected between 2008-2012, this revealed two additional SHUV cases to those previously published (van Eeden et al., 2012), both from equines in 2011. Collectively 9 cases of SHUV were identified in horses with neurological disease. Nucleotide alignment and P-distance analysis suggested very little intrinsic variation between the SHUV strains in the area selected for the diagnostic PCR, which is largely due to the high level of sequence conservation in the N gene of the orthobunyaviruses (Akashi et al., 1997; Saeed et al., 2000).

Annually, a high percentage of neurological disease in animals remains undiagnosed due to the substantial number of potential pathogens, which are excluded from routine diagnostics. The assay described provides a sensitive diagnostic tool, which will also be of use in epidemiological and surveillance studies, which will aid in the rapid identification of SHUV

in potentially undiagnosed cases of neurological disease leading to a broader understanding of neurological disease, especially in South Africa.

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