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Development of three triplex real-time reverse transcription PCR assays for the qualitative molecular typing of the nine serotypes of African horse sickness virus

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A B S T R A C T

Blood samples collected as part of routine diagnostic investigations from South African horses with clinical signs suggestive of African horse sickness (AHS) were subjected to analysis with an AHS virus (AHSV) group specific reverse transcription quantitative polymerase chain reaction (AHSV RT-qPCR) assay and virus isolation (VI) with subsequent serotyping by plaque inhibition (PI) assays using AHSV serotype-specific antisera. Blood samples that tested positive by AHSV RT-qPCR were then selected for analysis using AHSV type specific RT-qPCR (AHSV TS RT-qPCR) assays. The TS RT-qPCR assays were evaluated using both historic stocks of the South African reference strains of each of the 9 AHSV serotypes, as well as recently derived stocks of these same viruses. Of the 503 horse blood samples tested, 156 were positive by both AHSV RT-qPCR and VI assays, whereas 135 samples that were VI negative were positive by AHSV RT-qPCR assay. The virus isolates made from the various blood samples included all 9 AHSV serotypes, and there was 100% agreement between the results of conventional serotyping of individual virus isolates by PI assay and AHSV TS RT-qPCR typing results. Results of the current study confirm that the AHSV TS RT-qPCR assays for the identification of individual AHSV serotypes are applicable and practicable and therefore are potentially highly useful and appropriate for virus typing in AHS outbreak situations in endemic or sporadic incursion areas, which can be crucial in determining appropriate and timely vaccination and control strategies.

1. Introduction

African horse sickness (AHS) is an arboviral disease of horses that is endemic throughout much of sub-Saharan Africa, but significant incursions have occurred previously into North Africa, the Iberian Peninsula, the Middle East and the Indian subcontinent (Guthrie and Weyer, 2015; MacLachlan and Guthrie, 2010; Coetzer and Guthrie, 2004). Nine serotypes of AHS virus (AHSV) have been described (Howell, 1962; McIntosh, 1958). The genome of AHSV consists of 10 double stranded RNA segments, encoding 7 structural (VP1–VP7) and 4 non-structural (NS1, NS2, NS3/NS3A and NS4) proteins (Zwart et al., 2015; Roy et al., 1994; Grubman and Lewis, 1992). Genome segment 7 (S7) encodes the inner capsid protein VP7, which is highly conserved among the 9 AHSV serotypes (Quan et al., 2010) and is the basis for several antigen (Laviada et al., 1992; Hamblin et al., 1991), antibody (Maree and Paweska, 2005; Kweon et al., 2003; Wade-Evans et al., 1993; Hamblin et al., 1990), and nucleic-acid based diagnostic assays (Guthrie et al., 2013; Quan et al., 2010; Fernandez-Pinero et al., 2009; Agüero et al., 2008; Zientara et al., 1995). An outbreak of AHS in a naïve horse population can be devastating, with a cumulative mortality rate of up to 95% (Guthrie and Weyer, 2015; Coetzer and Guthrie, 2004; Mellor and Hamblin, 2004).

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A tentative diagnosis of AHS can be made on the basis of clinical signs and post mortem findings. Clinical signs considered indicative of AHS include those of pyrexia, supraorbital fossa swelling, subcutaneous oedema and respiratory distress due to pulmonary oedema (Guthrie and Weyer, 2015). These symptoms should be confirmed by laboratory diagnosis, as differential diagnoses such as Equine encephalosis virus infection must be ruled out. This has been done historically using virus isolation (VI), with subsequent determination of the serotype of individual virus isolates by virus neutralization assays (Guthrie and Weyer, 2015; Coetzer and Guthrie, 2004). This process is time-consuming, labour-intensive, and expensive. Several AHSV group-specific reverse transcription polymerase chain reaction (RT-PCR) assays have been developed recently for the diagnosis of AHS (Bachanek-Bankowska et al., 2014; Guthrie et al., 2013; Aradaib, 2009; Sailleau et al., 2000; Stone-Marschat et al., 1994). Advantages of AHSV RT-PCR assays are that they have the potential to be rapid, sensitive and versatile, and can supplement existing traditional virus identification methods. AHSV RT-PCR assays can also be applied to samples that do not contain infectious virus, or have very low viral titres, making them ideal screening tests (Quan et al., 2010). However, samples that are positive by RT-PCR, but negative by VI are common, especially amongst horses previously immunized against AHS with live-attenuated vaccine (Guthrie et al., 2013; Weyer et al., 2013). Determination of the serotype of AHSV contained in such samples currently requires further evaluation, with additional testing.

Determination of AHSV serotype is important in epidemiological studies and in order to quickly implement appropriate vaccination and control strategies. For example, outbreaks of AHS among horses in the AHS-controlled area in the Western Cape region of South Africa have highlighted the need for rapid and specific determination of the serotype of the incurring virus. Traditional VI and serotyping was used previously to identify the AHSV-1 serotype involved in an outbreak of AHS in the region in 2004 (Sinclair et al., 2006), whereas the AHSV-1 that was responsible for the outbreak in 2011 was determined by sequence analysis of the AHSV type-specific L2 (VP2) gene of the causative virus contained in the blood of an affected horse. This latter process took six days to complete before vaccination could be instituted (Grewar et al., 2013). AHSV type-specific real-time RT-PCR (TS RT-qPCR) assays offer the potential for more rapid determination of the AHSV type involved in such outbreaks. AHSV TS assays that target the portion of the L2 gene encoding the major neutralization determinants of AHSV (Kanai et al., 2014; Potgieter et al., 2003; Vreede and Huismans, 1994; Burrage et al., 1993) are well suited for accurate, rapid serotype determination. Direct TS RT-PCR assays have been developed (Sailleau et al., 2000); however these assays are cumbersome and time consuming as they require the use of gels for assay confirmation. Direct TS RT-PCR assays also increase the risk of contamination and false positive results. Koekemoer (2008) developed an AHSV TS RT-qPCR assay that utilizes a sensor probe and an anchor probe on two different channels, followed by melt curve analysis to differentiate the various AHSV types. Although these assays accurately confirmed the serotype of historic prototype South African strains of AHSV, they showed considerable variation in determination of the AHSV type of field strains. More recently, TS RT-qPCR assays for the detection and typing of AHSV have been developed and described (Bachanek-Bankowska et al., 2014).

The objective of the current study was to develop and characterize the applicability and practicability (Broeders et al., 2014) of AHSV TS RT-qPCR assays in a multiplex format (3 triplex assays) for the rapid molecular typing of samples determined to be positive for AHSV using a group specific RT-qPCR of documented diagnostic accuracy. Such assays will facilitate in the rapid determination of the virus type in field outbreaks of AHS thereby facilitating implementation of appropriate vaccination and control strategies in endemic areas such as South Africa. The applicability and practicability of these assays was evaluated when the assays were applied to nucleic acid extracted from blood samples collected from field cases of suspected AHS.

2. Materials and methods

2.1. Reference strains of AHSV

South African reference strains of the 9 AHSV serotypes were obtained in 1995 from the World Organization for Animal Health (OIE) AHS Reference Laboratory at the Onderstepoort Veterinary Institute (OVI). New reference strains of the 9 AHSV serotypes were obtained in 2014 from the same laboratory. Source data were not available for the 1995 reference strains. Details of the 2014 reference strains are provided in Table 1. The relationship between the two sets of reference strains is unknown. Reconstituted freeze-dried reference viruses obtained in both 1995 and 2014 were extracted and evaluated using the group specific AHSV RT-qPCR assay as previously described (Guthrie et al., 2013), followed by analysis with the AHSV TS-qPCR assays described below.

2.2. Field strains of AHSV

In a previous study (Guthrie et al., 2013), blood samples collected from pyrexic horses with signs typical of AHS (Guthrie and Weyer, 2015) between 1 January 2011 and 31 May 2012 were subjected to an AHSV group specific RT-qPCR assay and VI. These

Table 1
Information on the origin of the African horse sickness virus reference strains (2014); showing the name, isolate name and country of isolation with passage history and GenBank accession numbers for genes encoding VP2 of each virus.

Name	Isolate	Country	Passage History	GenBank Accession No.	
				ARC-OVI	ERC
AHSV1	HS29/62	South Africa	Mouse #2, BHK #2	KP939376	KT030571
AHSV2	HS82/61	South Africa	Mouse #2, Vero #1, BHK #2	KP939429	KT030581
AHSV3	HS13/63	South Africa	Mouse #3, Vero #1, BHK #2	KP939488	KT030591
AHSV4	HS32/62	Zimbabwe	Vero #1, BHK #2	KP939584	KT030601
AHSV5	HS30/62	South Africa	Mouse #2, BHK #2	KP939711	KT030611
AHSV6	HS39/63	South Africa	Vero #1, BHK #2	Not done	KT030621
AHSV7	HS31/62	South Africa	Mouse #1, Vero #2, BHK #2	KP939937	KT030641
AHSV8	HS10/62	Kenya	Mouse #1, Vero #1, BHK #2	KP940010	KT030651
AHSV9	HS90/61	Chad	Mouse #3, Vero #1, BHK #2	KP940141	KT030661

ARC-OVI, Agricultural Research Council–Onderstepoort Veterinary Institute; ERC, Equine Research Centre, University of Pretoria.