Serotype Specific Primers and Gel-Based RT-PCR Assays for ‘Typing’ African Horse Sickness Virus: Identification of Strains from Africa

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Abstract

African horse sickness is a devastating, transboundary animal disease, that is ‘listed’ by the Office International des Epizooties (OIE). Although attenuated, inactivated and subunit vaccines have been developed for African horse sickness virus (AHSV), these are serotype-specific and their effective deployment therefore relies on rapid and reliable identification of virus type. AHSV serotype is controlled by the specificity of interactions between neutralising antibodies, and components of the outer-capsid, particularly protein VP2 (encoded by AHSV genome segment 2 (Seg-2)). We report the development and evaluation of novel gel based reverse transcription-PCR (RT–PCR) assays targeting AHSV Seg-2, which can be used to very significantly increase the speed and reliability of detection and identification (compared to virus neutralisation tests) of the nine serotypes of AHSV. Primer sets were designed targeting regions of Seg-2 that are conserved between strains within each of the AHSV serotype (types 1 to 9). These assays were evaluated using multiple AHSV strains from the orbivirus reference collection at IAH (www.reoviridae.org/dsRNA_virus_proteins/ReoID/AHSV-isolates.htm). In each case the Seg-2 primers showed a high level of specificity and failed to cross-amplify the most closely related heterologous AHSV types, or other related orbiviruses (such as bluetongue virus (BTV), or equine encephalitis virus (EEV)). The assays are rapid and sensitive, and can be used to detect and type viral RNA in blood, tissue samples, or cultivated virus suspensions within 24 h. They were used to identify AHSV strains from recent outbreaks in sub-Saharan African countries. These methods also generate cDNAs suitable for sequencing and phylogenetic analyses of Seg-2, identifying distinct virus lineages within each virus-type and helping to identify strain movements/origins. The RT-PCR methods described here provide a robust and versatile tool for rapid and specific detection and identification of AHSV serotypes 1 to 9.

Introduction

African horsesickness (AHS) is an important transboundary animal disease that is ‘listed’ by the Office International des Epizooties (OIE). Although attenuated, inactivated and subunit vaccines have been developed for African horse sickness virus (AHSV), these are serotype-specific and their effective deployment therefore relies on rapid and reliable identification of virus type. AHSV serotype is controlled by the specificity of interactions between neutralising antibodies, and components of the outer-capsid, particularly protein VP2 (encoded by AHSV genome segment 2 (Seg-2)). We report the development and evaluation of novel gel based reverse transcription-PCR (RT–PCR) assays targeting AHSV Seg-2, which can be used to very significantly increase the speed and reliability of detection and identification (compared to virus neutralisation tests) of the nine serotypes of AHSV. Primer sets were designed targeting regions of Seg-2 that are conserved between strains within each of the AHSV serotype (types 1 to 9). These assays were evaluated using multiple AHSV strains from the orbivirus reference collection at IAH (www.reoviridae.org/dsRNA_virus_proteins/ReoID/AHSV-isolates.htm). In each case the Seg-2 primers showed a high level of specificity and failed to cross-amplify the most closely related heterologous AHSV types, or other related orbiviruses (such as bluetongue virus (BTV), or equine encephalitis virus (EEV)). The assays are rapid and sensitive, and can be used to detect and type viral RNA in blood, tissue samples, or cultivated virus suspensions within 24 h. They were used to identify AHSV strains from recent outbreaks in sub-Saharan African countries. These methods also generate cDNAs suitable for sequencing and phylogenetic analyses of Seg-2, identifying distinct virus lineages within each virus-type and helping to identify strain movements/origins. The RT-PCR methods described here provide a robust and versatile tool for rapid and specific detection and identification of AHSV serotypes 1 to 9.
Serotype-specific RT-PCR assays have been developed for the 26 serotypes of BTV [39,40,41]. Attempts to design similar primers and typing assays targeting Seg-2 of AHSV [42] have previously been hampered by a lack of full length Seg-2 sequence data for all AHSV serotypes. Generation of sequence data for Seg-2 from multiple reference and field strains of the nine serotypes of AHSV [31,34, Bankowska et al – manuscript in preparation] has clarified the level and extent of sequence variation within and between different serotypes.

We describe the design and in silico evaluation of Seg-2 based oligonucleotide primers and RT-PCR assays for each AHSV serotype. Assay specificity was evaluated with a range of AHSV isolates from dsRNA virus reference collection at IAH [44]. They were also used to identify AHSV strains circulating in East and West Africa, using RNA samples extracted directly from diagnostic blood or tissue samples. These techniques remove the need for virus isolation and ‘reference antisera’, increasing the speed and availability of virus identification methods, and reducing the use of experimental animals.

Materials and Methods

AHSV reference and field isolates

different AHSV isolates (listed in Table 1) from the Institute for Animal Health (IAH) ‘Oribivirus Reference Collection’ (ORC) [44] were grown in BHK-21 clone 13 (European Collection of Animal cell Cultures (ECACC – 84100501))/Vero (ECACC – 84113001) cell monolayers, until 40–100% cytopathic effect (CPE) was observed and in C. sonorensis (KC) cells (originally provided by colleagues at the USDA lab in Laramie, Wyoming) for 7 days. The field samples used in these studies were taken from naturally infected animals in the field, by qualified veterinarians, as part of normal veterinary care and diagnostic testing procedures in the respective countries. No samples were taken from animals specifically for these studies, so we believe that ethical approval is not required.

Extraction of Viral dsRNA

RNA was extracted from BTV infected cell pellets using Trizol® (Invitrogen) [45], or from cell-free tissue culture supernatant using the QIAamp Viral RNA Mini Kit (Qiagen), or a Universal BioRobot (Qiagen), according to manufacturer’s instructions.

Group specific RT-PCR assay

RNA samples extracted from clinical samples or cell culture material, were tested in duplicate by conventional RT-PCR assays using AHSV genome segment 7 (Seg-7) specific primers [46]. Samples that generated a strong cDNA band of the expected size (1179 Kb) were considered to contain AHSV RNA and were used for further processing/testing.

Serotype specific RT-PCR assays

Multiple primer-pairs were designed based on sequence data for Seg-2, targeting each of the nine AHSV serotypes (Table S1). cDNA copies were reverse transcribed from denatured dsRNA templates, then amplified by One-Step RT-PCR kit (Qiagen) as described by Mertens et al. [41], or by a one-step RT-PCR system (Invitrogen) and high fidelity platinum® Taq as described by Maan et al [47]. In some cases, full length cDNA copies of AHSV Seg-2 were also synthesised and sequenced, as described by Maan et al [48]. RT-PCR typing results were also confirmed by sequencing of amplified cDNA products from Seg-2 and sequence data analysed using MAFFT [49] and/or Clustal W programs [50]. Phylogenetic comparisons were made using Neighbour-Joining (NJ) algorithms and the trees were constructed in Mega 4.1 [51] using nucleotide
sequences in a pair-wise deletion, p-distance algorithm, and bootstrapped using 500 replicates.

### Results

**Development of RT-PCR assays and their evaluation for type specificity**

Comparisons of full-length Seg-2 sequences for reference strains of the nine AHSV serotypes and other field isolates [31,43,52,53, Bankowska et al – in preparation], were used to identify regions of conservation within individual AHSV serotypes and variability between different types. These regions were used to design a minimum of two serotype-specific primer-pairs for use in RT-PCR assays to detect each AHSV serotype (Table S1).

The RT-PCR primers were tested in reactions containing RNA extracted from BHK-21 cells infected with the different AHSV serotypes (Table 1). The cDNAs generated, were analysed by 1% agarose gel electrophoresis (AGE), to check both their size and

| Virus serotype<sup>1</sup> | Origin | IAH-P<sup>2</sup> Reference collection number | Passage history (MB<sup>4</sup>/BHK<sup>5</sup>) (V<sup>6</sup>/KC1<sup>7</sup>/?)<sup>8</sup> | Other details regarding samples |
|---------------------------|--------|-----------------------------------------------|-----------------------------------------------|-------------------------------|
| **AHSV Reference Strains** |        |                                               |                                               |                               |
| AHSV1                     | RSA<sup>2</sup> | RSArah1/03                                    | MB3/BHK10                                     |                               |
| AHSV2                     | RSA    | RSArah2/03                                    | MB3/BHK7                                      |                               |
| AHSV3                     | RSA    | RSArah3/03                                    | ?/BHK2                                        |                               |
| AHSV4                     | RSA    | RSArah4/03                                    | ?/BHK2                                        |                               |
| AHSV5                     | RSA    | RSArah5/03                                    | MB3/BHK7                                      |                               |
| AHSV6                     | RSA    | RSArah6/03                                    | MB3/BHK6                                      |                               |
| AHSV7                     | RSA    | KENrah7/03                                    | MB4/BHK9                                      |                               |
| AHSV8                     | RSA    | RSArah8/03                                    | ?/BHK2                                        |                               |
| AHSV9                     | RSA    | PAKrah9/03                                    | ?/BHK2                                        |                               |
| **AHSV Field Strains**    |        |                                               |                                               |                               |
| AHSV2                     | Ethiopia | ETH2010/01                                    | KC1                                            |                               |
| AHSV2                     | Senegal | SEN2007/01                                    | MB1/BHK2                                      | Sent from ‘The Republique du Senegal, Ministere de l’eleveage’ in May 2007 |
| AHSV2                     | Senegal | SEN2007/02                                    | MB1/BHK3                                      | Same as SEN2007/01 (see above) |
| AHSV2                     | Senegal | SEN2007/03                                    | MB1/BHK3                                      | From spleen of a six year old male horse, sampled on 26th August 2007 in Diourbel, Senegal. |
| AHSV2                     | Senegal | SEN2007/04                                    | MB1/BHK3                                      | From lung of a three year old female horse, sampled on 2nd September 2007 from Lerane Sambou village, Senegal. |
| AHSV2                     | Senegal | SEN2007/05                                    | MB1/BHK3                                      | From spleen of a six year old male horse, sampled on 24th August 2007 from Diongo village, Diourbel, Senegal. |
| AHSV4                     | Kenya   | KEN2007/01                                    | V3                                             | From a horse sampled in Kenya during October 2007 and passed on Vero cell culture at the Central Veterinary Research Laboratory (CVRL), Dubai. |
| AHSV4                     | Kenya   | KEN2007/02                                    | V2/KC1                                         | Same as KEN2007/01 (see above) |
| AHSV4                     | Spain   | SPA1987/01                                    | MB1/BHK3                                      |                               |
| AHSV6                     | Ethiopia | ETH2010/19                                    | KC1                                            |                               |
| AHSV7                     | Senegal | SEN2007/06                                    | V3                                             | From lung sample of a male horse from Guede Bousso village Senegal in September 2007 |
| AHSV7                     | Ethiopia | ETH2010/09                                    | V1                                             |                               |
| AHSV8                     | Ethiopia | ETH2010/10                                    | KC1                                            |                               |
| AHSV8 & 2                 | Ethiopia | ETH2010/23                                    | KC1                                            |                               |
| **AHSV9 vaccine**         | Senegal | SENvvvv/09                                     | ?/BHK2                                        | The Senegal AHSV-9 monovalent vaccine was identified as ‘an original vaccine lot M_0107, BEST BEFORE FEB 2009’ |

1Bankowska et al – in preparation;  
2RSA: Republic of South Africa;  
3Institute for Animal Health, Pirbright.  
4Number of passages in mouse brain;  
5Number of passages in BHK-21 cells;  
6Number of passages in Vero cells.  
7Number of passages in KC (Culicoides soronensis) cells;  
8Unknown.

IAH-P dsRNA virus reference collection number composed of ‘country code, year, and the number of the isolate in that year from that country. Full details of these isolates are available on the dsRNA virus reference collection website [44].

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relative level of synthesis. In each case cDNAs of the expected size were generated from RNA of the homologous serotype (Figure 1 panels A–D). None of the primer-pairs amplified cDNAs from RNA of even the most closely related heterologous AHSV types, or from members of other related Orbivirus species (data not shown). These initial evaluations confirm in silico analyses, indicating that the primers listed in Table S1 are indeed ‘type-specific’.

**Identification of AHSV-2 isolates from Senegal 2007.** RNA was extracted from equine samples, or from cell culture supernatants infected with these viruses (SEN2007/01, SEN2007/02, SEN2007/03, SEN2007/04 and SEN2007/05). In each case a strong +ve band of the expected size (1179 Kb) was generated in Seg-2 group-specific RT-PCR assays (Figure 2 – panel A). The RNA samples were also tested by conventional RT-PCR, using Seg-2 specific primers listed in Table S1. Strong +ve bands of the expected sizes were only generated with primer-pairs ‘2A1’ and ‘2A2’, demonstrating the presence of AHSV-2 RNA. None of the other AHSV types were detected, including AHSV-9 (which had recently been used to vaccinate the animals) (Figure 3). The AHSV-2 Seg-2 amplicons were sequenced, generating data for nucleotides (nt) 500–1640, which showed 97% nt identity with Seg-2 of the South African reference strain of AHSV-2 (Ac. No. AY163332) (Figure 4).

**Identification of AHSV-7 from Senegal 2007.** AHSV isolate (SEN2007/06) derived from a horse that died in 2007, was also tested in type-specific RT-PCR assays for each of the nine AHSV serotypes. Assays containing only one of the type 7 specific primers (‘7A1’) (Table S1) generated a band of the expected size (1426 bp) indicating that this sample contains AHSV-7 (Figure 5– panel C). Although the other type 7 specific primers (‘7A2’) worked well with the reference strain RSArah7/03 and Ethiopian strain (ETH2010/09), they failed to amplify sequences from SEN2007/06, suggesting a significant variation in the footprint sequence for this primer-pair compared to the reference strain (Table S1). The ‘7A1’ Seg-2 amplicon (nt 550–1800) from SEN2007/06, was also sequenced, showing 92% nt identity overall to Seg-2 of the South African AHSV-7 reference strain (Ac. No. AY163330) confirming the isolate as AHSV-7 (Figure 4).

**AHSV-9 vaccine strain from Senegal 2007.** The virus species of the original Senegal vaccine strain (SENvvvv/09) was confirmed (as AHSV) by the synthesis of an 1179 Kb cDNA in

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**Figure 1. ‘Type-specific’ primers for Seg-2 of AHSV-1 to AHSV-9.** The cDNAs amplified from Seg-2 of AHSV 1-9 serotypes using type-specific primers were analyzed by 1% agarose-gel-electrophoresis (AGE). **Panel A:** Lanes 1 and 2 show products from AHSV-1/RSArah1/03 using primer-pairs ‘1A1’ (1793 bp) and ‘1A2’ (1452 bp) respectively. Lanes 3 and 4: Primer-pairs ‘1A1’ and ‘1A2’ amplified only small amounts of ‘incorrectly’ sized products from AHSV-2/RSARah2/03 (heterologous-control). Lanes 5 and 6 show products from AHSV-2/RSARah2/03 using primer-pairs ‘2A1’ (1098 bp) and ‘2A2’ (1339 bp) respectively. Lanes 7 and 8: Primer-pairs ‘2A1’ amplified none but ‘2A2’ amplified only small amounts of ‘incorrectly’ sized products from AHSV-3/RSARah3/03 (heterologous-control). Lanes 9 and 10: Primer-pairs ‘3A1’ (751 bp) and ‘3A2’ (1524 bp) respectively. Lanes 11 and 12: Primer-pairs ‘3A1’ and ‘3A2’ showed no amplification from AHSV-7/KENhrah7/03 (heterologous-control). **Panel B:** Lanes 1 and 2 show products from AHSV-4/RSARAH4/03 using primer-pairs ‘4A1’ (1264 bp) and ‘4A3’ (1471 bp) respectively. Lanes 3, 4, 5 and 6: Primer-pairs ‘4A1’ and ‘4A3’ did not amplify Seg-2 from AHSV-5/RSARah5/03 and AHSV-8/RSARah8/03 respectively (heterologous-control). Lanes 7 and 8 show products from AHSV-5/RSARah5/03 using primer-pairs ‘5A2’ (1423 bp) and ‘5A3’ (1139 bp) respectively. Lanes 9, 10, 11 and 12: Primer-pairs ‘5A2’ and ‘5A3’ did not amplify Seg-2 from AHSV-7/KENhrah7/03 and AHSV-8/RSARah8/03 respectively (heterologous-control). **Panel C:** Lanes 1 and 2 show products from AHSV-6/RSARah6/03 using primer-pairs ‘6A1’ (1154 bp) and ‘6A2’ (1270 bp) respectively. Lanes 3 and 4: Primer-pairs ‘6A1’ and ‘6A2’ amplified only small amounts of ‘incorrectly’ sized products from AHSV-8/RSARah8/03 (heterologous-control). Lanes 5 and 6 show products from AHSV-7/KENhrah7/03 using primer-pairs ‘7A1’ (1426 bp) and ‘7A2’ (1426 bp) respectively. Lanes 7 and 8: Primer-pairs ‘7A1’ and ‘7A2’ amplified only small amounts of ‘incorrectly’ sized products from AHSV-3/RSARah3/03 (heterologous-control). **Panel D:** Lanes 1 and 2 show products from AHSV-8/RSARah8/03 using primer-pairs ‘8A1’ (1757 bp) and ‘8A2’ (1732 bp) respectively. Lanes 3, 4, 5 and 6: Primer-pairs ‘8A1’ and ‘8A2’ did not amplify Seg-2 from AHSV-4/RSARah4/03 and AHSV-5/RSARah5/03 respectively (heterologous-control). Lanes 7 and 8 show products from AHSV-9/PAKrah9/03 using primer-pairs ‘9A1’ (1483 bp) and ‘9A2’ (1706 bp) respectively. Lanes 9 and 10: Primer-pairs ‘9A1’ and ‘9A2’ did not amplify Seg-2 from AHSV-6/RSARah6/03 (heterologous-control). Lane M: 1 Kb DNA marker (Invitrogen). doi:10.1371/journal.pone.0025686.g001
group-specific RT-PCR assays containing Seg-7 primers (Figure 2). In subsequent type-specific assays, only two primer sets targeting Seg-2 of AHSV-9 (‘9A1’ and ‘9A2’) (Table S1) generated strong cDNA bands of the expected size, indicating that the vaccine did not contain other AHSV types (Figure 3 – panel D). The Seg-2 amplicons were sequenced (nt 520–1684) showing 98.8% nt identity with Seg-2 of the South African AHSV-9 reference strain (Ac. No. AF043926) confirming the virus type (Figure 4).

AHSV-4 isolates from Kenya 2007. RNA extracted from Kenyan isolates KEN2007/01 and KEN2007/02 were tested in conventional type-specific RT-PCR assays targeting Seg-2 (Figures 4 and 5). Only primer-pairs ‘4A1’, ‘4A2’ and ‘4A3’ generated strong PCR amplicons of 1179 bp were generated from AHSV Seg-7 specific RT-PCR of dsRNA extracted from equine lung and spleen extracts and vaccine strain from Senegal tested in duplicate (SEN2007/01 – lanes 1 and 2; SEN2007/02 – lanes 3 and 4 in panel A; SENvvvv/09 – lanes 1 and 2 in panel B respectively) [46], demonstrating that the samples contain AHSV RNA. Lane – C is a negative water control showing no amplification. Lane M: 1 Kb marker (Invitrogen). RNA from BTV-4/RSArrrr/04 was used as a positive control using primer-pair ‘4W2’ - 2324 bp [41] (lane +C, panel B).

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Figure 2. Electrophoretic analysis of cDNA products generated in AHSV group-specific RT-PCR assays using Seg-7 specific primers. Panel A and B: PCR amplicons were generated from AHSV Seg-7 specific RT-PCR of dsRNA extracted from equine lung (SEN2007/01 – panel A) and spleen (SEN2007/02 – panel B) using primer-pairs ‘2A1’ (1098 bp, lane 5 in both panels) and ‘2A2’ (1339 bp, lane 6 in both panels) (Table S1), demonstrating that the samples contain AHSV-2 RNA. Lanes 3–20 represent RNA tested from AHSV-1 to 9 using two pairs of primers for each serotypes. Lane 1 is a negative water control showing no amplification. Lane M: 1 Kb marker (Invitrogen). RNA from BTV-4/RSArrrr/04 was used as a positive control using primer-pair ‘4W2’ - 2324 bp [41] (lane 2).

Panel C: PCR amplicons were generated from AHSV Seg-2 specific RT-PCR of dsRNA extracted from SEN2007/06 using primer-pairs ‘7A1’ (1426 bp - lane 7) (Table S1), demonstrating that the samples contain AHSV-7 RNA. Lanes 1–9 represent RNA tested from AHSV-1 to 9 using first set of primers for each serotypes. No amplification was detected in other serotypes. Lane – C is a negative water control showing no amplification. Lane M: 1 Kb marker (Invitrogen). RNA from ASHV multivalent vaccine strain (SENvvv1/MV) with ‘3A1’ was used as positive control which generated a product of 751 bp (lane +C). Panel D: PCR amplicons were generated from AHSV Seg-2 specific RT-PCR of dsRNA extracted from SENvvv/09 using primer-pairs ‘9A1’ (1483 bp - lane 18) and ‘9A2’ (1706 bp – lane 19) (Table S1), demonstrating that this sample contains AHSV-9 RNA. Lanes 3–20 represent RNA tested from AHSV-1 to 9 using two pairs of primers for each serotypes. Lane 1 is a negative water control showing no amplification. Lane M: 1 Kb marker (Invitrogen). RNA from BTV-4/RSArrrr/04 was used as a positive control using primer-pair ‘4W2’ - 2324 bp [41] (lane 2).

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Figure 3. Electrophoretic analysis of cDNA from Seg-2 of AHSV isolates from Senegal 2007 using ‘type-specific’ primer-pairs. Panel A and B: PCR amplicons were generated from AHSV Seg-2 specific RT-PCR of dsRNA extracted from equine lung (SEN2007/01 – panel A) and spleen (SEN2007/02 – panel B) using primer-pairs ‘2A1’ (1098 bp, lane 5 in both panels) and ‘2A2’ (1339 bp, lane 6 in both panels) (Table S1), demonstrating that the samples contain AHSV-2 RNA. Lanes 3–20 represent RNA tested from AHSV-1 to 9 using two pairs of primers for each serotypes. Lane 1 is a negative water control showing no amplification. Lane M: 1 Kb marker (Invitrogen). RNA from BTV-4/RSArrrr/04 was used as a positive control using primer-pair ‘4W2’ - 2324 bp [41] (lane 2).
bands of the expected sizes (1264 bp, 1463 bp and 1471 bp respectively) identifying the virus as AHSV-4. This indicates that these isolates did not contain RNA from other recognised AHSV serotypes. The Seg-2 amplicons were sequenced, showing 96% nt identity with Seg-2 of the South African AHSV-4 reference strain (Ac. No. M90697) confirming the virus type (Figure 4).

**Discussion**

AHSV Seg-2 encodes VP2 (the main serotype-specific antigen and most variable of the virus proteins) and shows 47.6% to 71.4% nt identity between types, which can therefore be identified by phylogenetic comparisons [31,43, Bankowska et al – in preparation]. Cross-hybridization methods [54] or hybridization and real-time RT-PCR methods targeting AHSV Seg-2 have previously been described [55,56]. Sailleau, et al [42] have also reported typing of AHSV using a serogroup-specific forward-primer and a serotype-specific reverse-primer, based on a limited Seg-2 dataset. These primer-pairs generated a small amplicon for each of nine serotypes of AHSV and were used to ‘type’ a limited number of reference and field strains. The RT-PCR assay results obtained were compared with VNT results but were not confirmed by sequencing of the resulting amplicons.

Recent full length sequencing of Seg-2 from reference strains and multiple field strains of AHSV (Bankowska et al – in preparation), have supported the design of additional AHSV ‘type-specific’ primer-pairs. We have successfully evaluated these primers, RT-PCR assays, sequencing and phylogenetic analyses, for differentiation of individual AHSV strains even within a single serotype. These techniques can determine AHSV serotype with greater accuracy, sensitivity and significantly more quickly than is possible by current serological techniques, facilitating surveillance and molecular epidemiology studies of the virus.

The relatively small number of AHSV isolates (N = 26 (Table 1); N = 107 in silico (Table S1)) that are available for sequence analyses and testing, has limited wider validation of the assays developed reported here. The specificity of the primers therefore primarily reflects their failure to recognise and amplify Seg-2 of other AHSV serotypes, rather than an ability to detect all possible isolates of the homologous type. If in future studies, any of these primers fail to detect Seg-2 of a novel isolate, it will be sequenced, providing a basis for further ‘evolution’ of primer sequences and assays. The ‘fine-scale’ differentiation and identification of individual AHSV strains, depends on the availability and characterisation of well documented reference isolates from different locations and belonging to different virus lineages. These need to be stored and maintained in a suitable reference collection (like the ORC) with links to relevant sequence data [44].

Recent disease outbreaks caused by AHSV-4 in Kenya (in 2007), AHSV-2, AHSV-7 and AHSV-9 in Senegal (in 2007), were identified using the ‘type-specific’ assays (as described here). Serotype 9 was also identified in the Gambia (in 2009) along with multiple serotypes in Ethiopia (in 2010) using the same techniques [15], Bankowska et al, in preparation. These novel laboratory
tools and the standardised protocol are easy to follow and do not require reference antisera or virus isolates that may have limited availability and which themselves represent a potential disease security risk. These methods are robust and versatile, reliably identifying AHSV type within 24 h of sample receipt, with no evidence of cross-amplification of other related types or other orbiviruses. Although slower than real-time RT-PCR assays, these conventional type-specific primers and assays can be used to generate cDNA amplicons for sequence analyses, providing definitive proof of relationships between Seg-2 of individual virus strains.

These phylogenetic comparisons will help to determine the prevalence, distribution and movements of different AHSV serotypes and topotypes, improving our understanding of the epidemiology and transmission of the virus, providing a basis for the design and rapid implementation of control strategies, including vaccination.

Supporting Information

**Table S1 Primers for specific amplification of Seg-2 from various AHSV serotypes in RT-PCR assays.**

(DOC)

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Author Contributions

Conceived and designed the experiments: NSM PPCM SM. Performed the experiments: NSM SM KN KB-B. Analyzed the data: NSM SM KN KB-B MNB. Contributed reagents/materials/analysis tools: NSM SM PPCM KB-B MNB. Wrote the paper: NSM PPCM SM.

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