Molecular and serological surveillance of African horse sickness virus in Eastern and Central Saudi Arabia

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Summary

African horse sickness virus (AHSV) is one of the most devastating viral diseases of the family *Equidae*. Infection with AHSV threatens not only the Saudi equine industry but also the equine industry worldwide. This is due to the high morbidity and mortality rates among the infected population of up to 100%. The World Organisation for Animal Health (OIE) lists AHSV among its notifiable diseases; this requires Member Countries to monitor the situation with regard to AHSV very carefully in order to avoid the spread of the virus. The OIE also suggests the systematic monitoring of AHSV in the equine population at regular intervals. The main aim of the current study is to perform molecular and serological surveillance on different horse populations in Eastern and Central regions of Saudi Arabia. To achieve this aim, the authors collected 361 serum samples, 103 whole blood samples and 323 swabs from Al-

Hasa, Dammam, Al-Jubail, Al-Qateef, Riyadh and Al-Qassim. Commercial enzyme-linked immunosorbent assay (ELISA) kits were used to detect AHSV antibodies and commercial real-time reverse transcriptase-polymerase chain reaction (RT-PCR) kits were used to detect AHSV nucleic acids in blood and swabs. The results of this study demonstrate the absence of anti-AHSV antibodies in the sera of tested animals. Furthermore, no viral nucleic acids were detected in the collected blood and swab samples, as evaluated by real-time AHSV-RT-PCR. Moreover, all tested samples collected during 2014–2016 were negative for AHSV. This confirms that the horse populations studied in the Eastern and Central regions of Saudi Arabia during 2014–2016 were AHSV free.

Keywords

African horse sickness virus – Central – Eastern – ELISA – Real-time RT-PCR – Saudi Arabia.

Introduction

African horse sickness virus (AHSV) is one of the most important viral threats to the family *Equidae*, which includes horses, ponies, mules and donkeys (1). It was reported in Yemen for the first time in 1327. However, it is believed that the virus originated in Africa and spread to Asia through the transfer of wild animals, such as zebras. Several outbreaks have been reported on a regular basis in South Africa, and other African countries have reported AHSV outbreaks over the years (2).

African horse sickness virus belongs to the family *Reoviridae* and genus *Orbivirus*. This genus includes many important viruses affecting several animal species, including Bluetongue virus in sheep, Epizootic haemorrhagic disease virus (EHDV) and Equine encephalosis virus (EEV) (3, 4). It has been reported that donkeys and wild equids (e.g. zebras) are AHSV reservoirs (5). The AHSV genome consists of ten segments of double-stranded ribonucleic acid (dsRNA) surrounded by a multilayered capsid. The outermost layer of the capsid consists mainly of two viral proteins (VP2 and VP5), which are

responsible for the virus's attachment to its host's cell membranes and cell entry (6). The viral genome encodes several structural proteins (VP1–VP7) and non-structural proteins (NS1, NS2, NS3 and NS3A). The most conserved protein in all serotypes is VP7 (7). The virus can cause four forms of the disease, including cardiac, pulmonary, mixed forms and horse sickness fever (8). It is one of the arboviruses transmitted by midges. During cooler periods, for example in the winter months, the virus can remain latent through dormant maintenance in the fly followed by reactivation when the temperature increases in the spring. Vertical transmission in flies is not demonstrated for AHSV or for the more frequently studied Bluetongue virus (9). Nine serotypes of AHSV have been identified. Serotypes 2, 7 and 9 have been reported in North and West Africa, allowing for further transmission to neighbouring Mediterranean countries (10). Although the virus neutralisation test was previously the gold standard test for the serotyping of AHSV, the newly developed reverse transcriptase-polymerase chain reaction (RT-PCR) and real-time RT-PCR analyses provide rapid, sensitive and accurate tools for the identification of AHSV genotypes (10, 11). The isolation of AHSV can be performed during the acute febrile stage of the disease from the blood of infected animals. The virus can also be isolated from the spleen, lungs, and lymph nodes (12). One of the main goals of the current study is to clarify the dilemma regarding the presence/absence of AHSV in Saudi Arabia using the most recent state-of-the-art molecular virology and serological techniques.

Several outbreaks were reported in the Middle East during the period of 1959–1963 (13, 14, 15). However, in the Gulf countries, research was conducted 29 years ago that documented the absence of AHSV in Saudi Arabia. These studies relied on the use of historical serological techniques such as the agar gel immunodiffusion test (13). However, another study was conducted in Qatar during 1990 that reported the presence of AHSV serotype 9 in two seven-year old Arabian horses (14). An investigation into this outbreak was conducted via virus isolation in suckling mice and serology using the complement fixation test (CFT). These horses had recently been vaccinated against AHSV with a commercially available inactivated vaccine (14). Meanwhile,

antibodies against AHSV had been reported in horses in some other Gulf countries such as Oman in the early 1980s (15). Another serosurveillance study was conducted after the 1989 AHSV outbreak in Saudi Arabia to investigate the presence of AHSV in horses and donkeys in the southern part of Saudi Arabia (16). This study reported that more than 30% of the sera tested from horses and donkeys were positive for AHSV using the standard blocking African horse sickness virus-enzyme-linked immunosorbent assay (AHSV-ELISA) technique. The study's authors suggested that the virus had been introduced to Saudi Arabia by donkeys crossing the border from Yemen. Some recent studies have shown that AHSV still actively circulates in Africa, with recent outbreaks of AHSV reported in Namibia and in South Africa. In Namibia, several strains of the virus were isolated, such as serotypes 1, 2, 4, 6, 7, 8 and 9, and were found to be closely related to those strains previously characterised in South Africa (17). A recent study was conducted in France to evaluate the risk of the AHSV spreading to the country through the introduction of the virus's vectors (the *Culicoides* species) – this type of midge has been previously reported as introducing the Bluetongue virus to France. Moreover, the study warned of a higher seasonal risk of AHSV introduction through infected vectors (18). A report published by the Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture (USDA) in the same year (2015) concluded that Saudi Arabia was AHSV free (19).

Materials and methods

Area of study and animals

The current study focuses on two major regions of Saudi Arabia with dense horse populations: the Eastern and Central regions. The authors selected several stables in Al-Hasa, Dammam, Al-Jubail and Al-Qateef in the Eastern region. Furthermore, they also selected horse populations from the areas of Riyadh and Al-Qassim representing the Central region (Table I). Different categories of horse management were included in the sampling. Some of the study's animals had taken part in local horse races and shows; others had been used by farmers

as heavy-duty animals. Moreover, some of the study's animals were in close contact with other animals, such as camels, cattle, sheep, goats, chickens, etc.

Animal ethics statements

All animal experiments and sample collections were conducted as per the guidelines on implementing relevant regulations drafted by the King Abdulaziz City of Science and Technology National Committee of Bio Ethics (NCBE) (20). In addition, the study's animal utilisation protocol was amended by NCBE.

Sample collection and processing

Serum samples

Serum samples (361) were collected from different regions in Eastern and Central Saudi Arabia (Table I and Fig. 1). These samples were collected from January 2014 to December 2016. All animals used in this study were in good health showing no obvious clinical signs of any disease. The animals were selected to include both sexes and different ages. Moreover, the animals used in these surveillances represented different management systems such as racing, farming and sentinel herds. No animals were vaccinated against AHSV. Blood samples were collected by venepuncture from the jugular vein and were kept overnight at 4°C. The collected blood samples were centrifuged at 1957 g for 10 min and the serum transferred to new tubes. Serum samples were heat-inactivated at 56°C for 30 min and were stored at –20°C for further testing.

Swabs

Nasal and rectal swabs (323 of each) were collected from animals in the study from January 2014 to December 2016. Table I shows the number of each type of swab collected in the different regions of Eastern and Central Saudi Arabia included in the study (Fig. 1). The samples were collected by immersing the tip of each swab into either the nasal or rectal opening of each animal, independently. The collected swabs were transferred to viral transport media containing

(Dulbecco's Modified Eagle's Medium (DMEM), penicillin, streptomycin, 5% foetal bovine serum). The authors processed the swabs by maceration of the cotton pieces against the wall of the tube. The collected swabs were centrifuged at 1957 g for 10 min at 4°C and the resulting supernatant was stored at –80°C for further testing.

Whole blood

The whole blood samples (103) were collected from animals via venepuncture. Blood was collected in tubes containing ethylene diamine tetra-acetic acid (EDTA), and gentle shaking was applied to mix the whole blood with the EDTA. Samples were transferred on ice to the authors' laboratory. The whole blood was processed as previously described by Oladosu *et al*. (21). Specifically, the whole blood was centrifuged at 1957 g for 5 min at 4°C. Next, the buffy coat was carefully collected by pipetting. Finally, these buffy coats were kept at –80°C for downstream testing using molecular techniques.

Ribonucleic acid extraction

All viral ribonucleic acid (RNA) was extracted from the collected swabs and EDTA–blood solution using QIAamp® Viral RNA Mini Kit (Qiagen, Hilden, Germany) as per the kit instructions. The RNA concentration was measured using a Thermo Scientific™ NanoDrop 2000 spectrometer, and the RNA samples were stored at –80°C until testing.

African horse sickness virus group real-time reverse transcriptase-polymerase chain reaction

The RNA extracted from the collected swabs was evaluated for AHSV RNA using a commercial real-time RT-PCR kit (Path-AHSV-standard, Primerdesign Ltd, Chandler's Ford, United Kingdom). The real-time RT-PCR technique was conducted according to the manufacturer's instructions. Initially, the dsRNA was denatured as per Aklilu *et al*. (22). Specifically, the authors prepared a 15µl reaction solution, including 10µl of Oasig™ Lyophilised OneStep qRT–PCR Mastermix, 1µl of AHSV probe and 4µl of RNAse free water. The

amount of 15µl of the master mix was transferred to each well of the real-time RT-PCR plates. To this, 5µl of each RNA sample was added to the assigned well. The conditions for the RT-PCR reaction were: reverse transcription at 55°C for 10 min, activation of the enzymes at 95°C for 2 min, denaturation at 95°C for 10s then data collection at 60°C for 60s, repeated for 50 cycles. The test was deemed valid when the positive control generated a cycle threshold (Ct) between cycles 16 and 23. The tested sample was considered positive if its Ct <35. The authors carried out this test in an Applied Biosystems® 7500/7500 Fast Real-Time PCR System thermal cycler (Applied Biosystems Inc., California, United States).

Synthesis of African horse sickness virus complementary DNA and the polymerase chain reaction

The extracted RNA samples were subjected to two-step RT-PCR. The technique was carried out according to Stone-Marschat *et al.* (23), with some modifications. The RT-PCR reactions were performed in a 20µl reaction solution, including 2µl of dsRNA samples, 1µl of sense AHSV primer (23), 1µl of Moloney murine leukemia virus reverse transcriptase (MMLV RT, TakaRa, Beijing, People's Republic of China [China]). The synthesised complementary DNA (cDNA) was amplified by the RT-PCR. Reaction solutions of 50µl were prepared containing 1µl of each template cDNA, both AHSV sense and antisense primers, PCR master mix and 1µl of Taq DNA polymerase (TakaRa, China). The authors used the following parameters (initial denaturation for 5 min at 95°C, then 94°C for 1 min, then annealing at 55°C for 30 sec repeated for 30 cycles, with the final extension at 72°C for 10 min).

Gel electrophoresis

The authors separated 10µl of each amplified RT-PCR reaction using 1% agarose gels containing SYBR® Safe DNA Gel Stain (Life Technologies, Grand Island, New York, United States). The reactions were visualised under ultraviolet light and the gels were photographed using the Gel Doc™ XR Gel Documentation System (Bio-Rad Laboratories Inc., Hercules, California, United States).

Enzyme-linked immunosorbent assay

The sera collected from the animals were tested in duplicate using commercially available ELISA kits (Ingezim AHSV Compac Plus 14. AHS. K. 3, Catalogue No.: S0812) according to the kit instructions and as previously described in Ehizibolo *et al*. (24). The ELISA plates' optical density (OD) was measured at 405nm using a BioTek Synergy™ Mx Microplate Reader (Winooski, United States). The optical density data was interpreted by applying the following formula: blocking percentage (BP) = (negative OD – sample OD) / (negative OD – positive OD). A sample was considered to be positive when the BP was greater than or equal to 50%.

Results

Evaluation of the antibody response of horses to African horse sickness virus in Eastern and Central Saudi Arabia

All the serum samples collected from the Eastern and Central regions of the kingdom were tested for antibodies against AHSV (Table I). First, the authors checked the validity of the ELISA procedure as per the kit's instructions. Then, they tested all the collected sera and found that all the tested samples were AHSV negative, according to the calculation methods recommended for use with the kits (Table II).

Molecular surveillance of African horse sickness virus in Eastern and Central Saudi Arabia

Molecular surveillance was conducted by sampling different horse populations in the Eastern and Central regions of Saudi Arabia (Table III). This surveillance study was conducted from November 2014 to October 2016. All tested nasal swabs, whole blood and rectal swabs (data not shown), were found negative for AHSV (Table II, Fig. 2a). Furthermore, 103 whole blood samples were evaluated for AHSV using real time RT-PCR. All the tested whole blood samples collected were negative for AHSV (Table IV and Fig. 2b). For further confirmation, the nasal and rectal swabs were tested using the regular RT-PCR technique targeting Segment 8, which encodes the NS2 gene

(Fig. 3). The results clearly show an absence of AHSV in the tested whole blood; and nasal and rectal swabs from the horses from different regions in Eastern and Central Saudi Arabia tested in this study.

Discussion

African horse sickness is one of the most devastating viral diseases of the equine species, which includes horses, donkeys, ponies, mules and zebras. It was the first viral candidate affecting the *Equidae* family to be listed in the OIE notifiable disease report (25). Although AHSV is endemic in Africa (e.g. Senegal, South Africa, Sudan, Morocco, etc. [26, 27, 28, 29]) several outbreaks have been reported in many other parts in the world (e.g. Spain [30]). Moreover, AHSV was detected in many countries in the Middle East (13, 14). In the Gulf countries, some research claimed AHSV was endemic (13, 14, 15). Meanwhile, AHSV was reported in two horses from Qatar in 1990. Those two animals received formalin inactivated AHSV vaccine. The identity of the virus was confirmed via virus isolation, the complement fixation test (CFT), and the serum neutralisation test (SNT). It was postulated that the animals acquired the infection from the residual active live viruses in the inactivated vaccine administered to them ten days prior to the onset of the disease (14). Furthermore, another study reported the presence of AHSV antibodies in the sera of some animals in Oman (15). In addition, one study of Saudi Arabia reported the presence of AHSV in some animals in 1989 (16). However, some other studies reported the absence of AHSV in horses based on the clinical inspection of animals, virus isolation and serological surveillance during 1992–1995 (13). Due to Saudi Arabia's open borders with Yemen and the close proximity to Djibouti, considered an AHSV endemic area, the OIE suggested the continuous monitoring of AHSV in Saudi Arabia. A recent study from the USDA-APHIS revealed the absence of AHSV in Saudi Arabia (19). The current study's serological and molecular surveillance results are consistent with the most recent USDA report and support the conclusion that Eastern and Central regions of Saudi Arabia are free of AHSV. The authors tested nasal and rectal swabs, and whole blood samples for the presence of

AHSV in specimens collected from horses in Eastern and Central Saudi Arabia between 2014 and 2016 (Figs 2 and 3). It is well known that whole blood and infected tissues (spleen, lungs and lymph nodes) are the ideal samples for testing for the presence of AHSV in horses, while no virus excretion has ever been demonstrated in nasal discharge or in faeces. In addition, the authors tested nasal and rectal swabs of horses for the presence of AHSV nucleic acids using real time RT-PCR (Tables I–IV and Figs 2–3) and the data show the absence of AHSV in all tested swabs (Figs 2–3). Furthermore, selected nasal and rectal swabs also tested negative using conventional RT-PCR (Fig. 3). Continued vigilance and molecular monitoring is required along with the surveillance of clinical disease in horses and serosurveillance in order to maintain freedom from AHSV since it has the ability to spread rapidly.

Conclusions

The results clearly show the absence of both antibodies and viral nucleic acids of AHSV in blood and nasal and rectal swabs from horses in Eastern and Central regions of Saudi Arabia. This confirms that horses in these regions were free from AHSV during the period of the current study (2014–2016).

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Table I

Summary of the collected samples in the current study and their geographical locations

N	Region	Number of collected samples			
		Nasal swabs	Rectal swabs	Whole blood	Sera
1	H	60	60	64	60
2	Qt	104	104	0	130
3	D	44	44	6	52
4	J	49	49	0	53
5	R	47	47	14	47
6	Qs	19	19	19	19
Total		323	323	103	361

D: Dammam

H: Al-Hasa

J: Al-Jubail

Qs: Al-Qassim

Qt: Al-Qateef

R: Riyadh

Table II

Summary of the enzyme-linked immunosorbent assay results for African horse sickness virus in horses in Eastern and Central Saudi Arabia, 2014–2016

N	Region	Number of AHSV ELISA samples		
		Tested	+ve	–ve
1	H	60	0	60
2	Qt	130	0	130
3	D	52	0	52
4	J	53	0	53
5	R	47	0	47
6	Qs	19	0	19
Total		361	0	361

AHSV: African horse sickness virus

D: Dammam

ELISA: Enzyme linked immunosorbent assay

H: Al-Hasa

J: Al-Jubail

Qs: Al-Qassim

Qt: Al-Qateef

R: Riyadh

Table III

Summary of the real time reverse transcriptase-polymerase chain reaction results for African horse sickness virus in horses in Eastern and Central Saudi Arabia, 2014–2016

N	Region	Number of swabs				
		Tested	Nasal		Rectal	
			+ve	–ve	+ve	–ve
1	H	60	0	60	0	60
2	Qt	104	0	104	0	104
3	D	44	0	44	0	44
4	J	49	0	49	0	49
5	R	47	0	47	0	47
6	Qs	19	0	19	0	19
Total		323	0	323	0	323

D: Dammam

H: Al-Hasa

J: Al-Jubail

Qs: Al-Qassim

Qt: Al-Qateef

R: Riyadh

Table IV

Summary of the real time reverse transcriptase-polymerase chain reaction results on whole blood for African horse sickness virus in horses in Eastern and Central Saudi Arabia, 2014–2016

N	Region	Number of samples		
		Tested	Whole blood	
			+ve	–ve
1	H	64	0	64
2	Qt	0	0	0
3	D	6	0	6
4	J	0	0	0
5	R	14	0	14
6	Qs	19	0	19
Total		103	0	103

D: Dammam

H: Al-Hasa

J: Al-Jubail

Qs: Al-Qassim

Qt: Al-Qateef

R: Riyadh

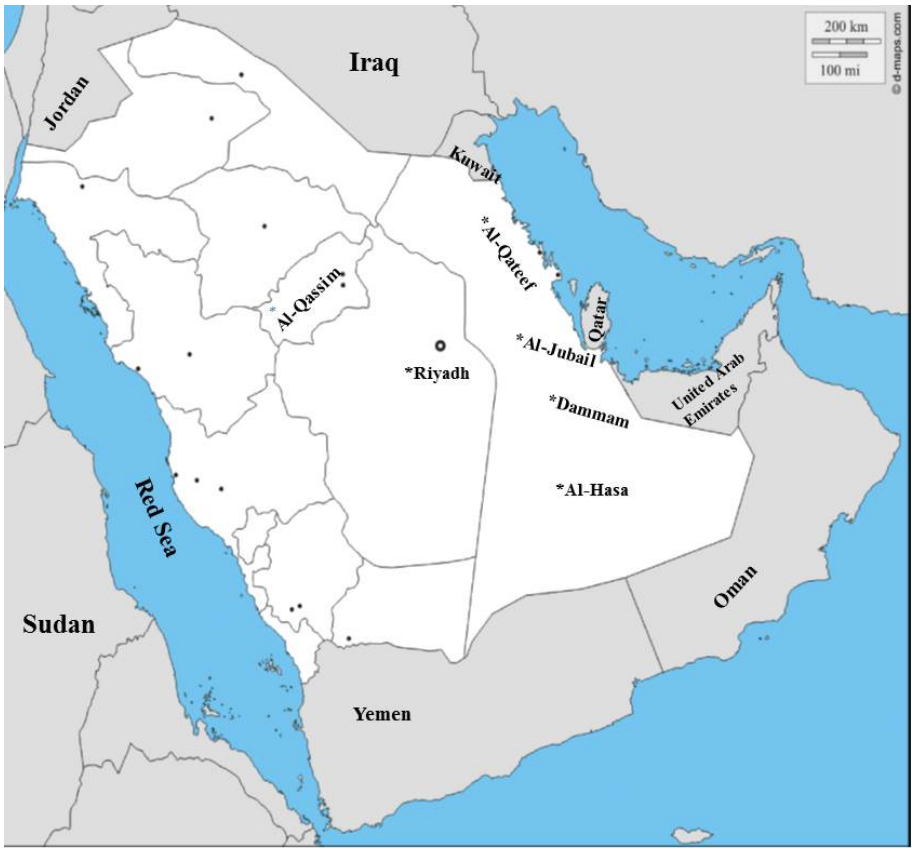
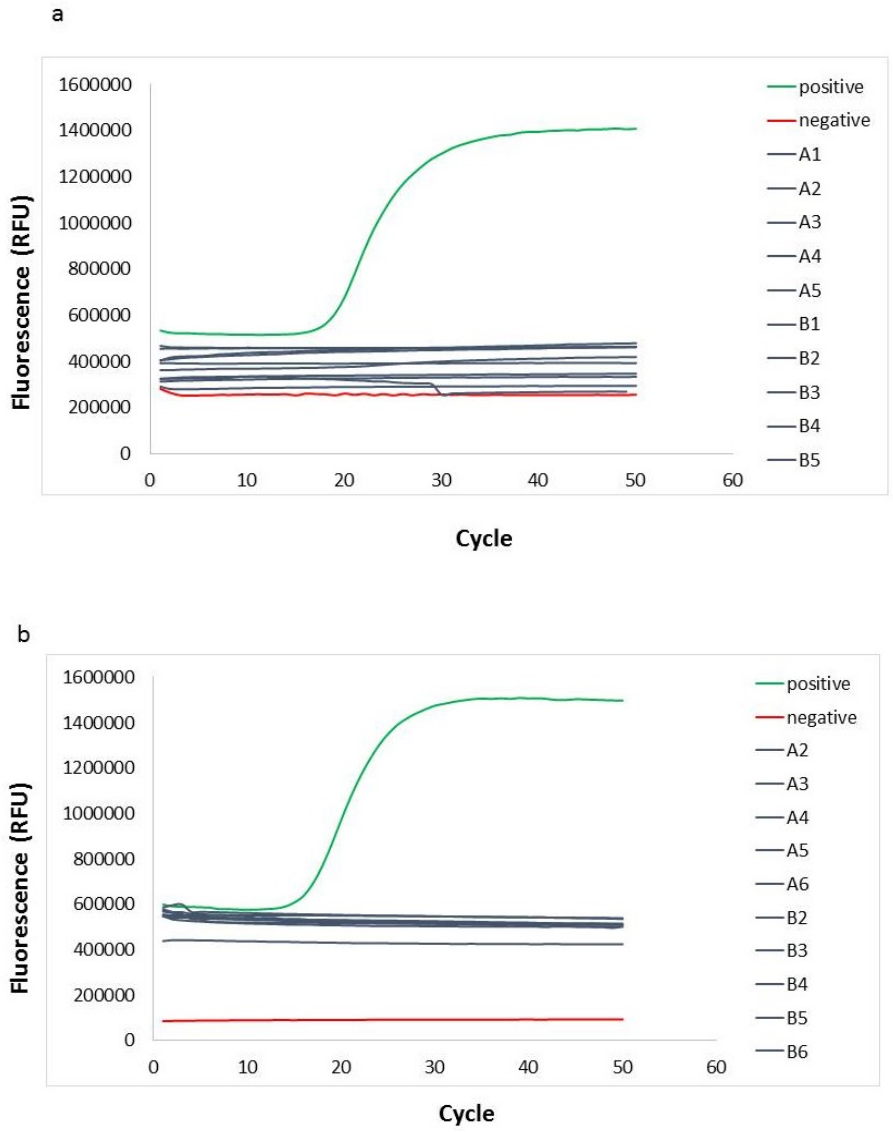


Fig. 1
Map of Saudi Arabia showing the geographical distribution of the collected samples

* indicates the locations from which the samples were collected



A2–B6: nasal swab specimens

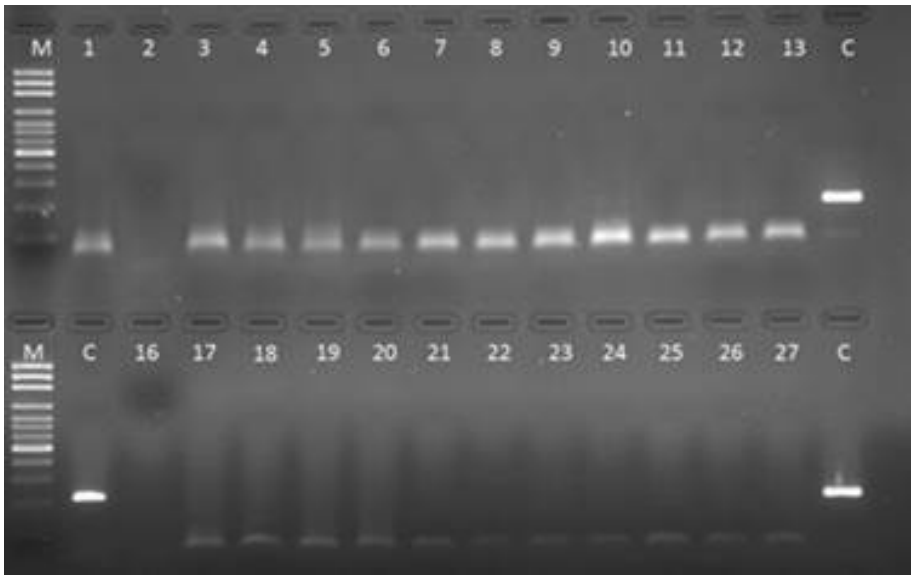
A1–B5: whole blood specimens

RFU: relative fluorescence unit

Fig. 2

Real time reverse transcriptase-polymerase chain reaction amplification curves for the (a) nasal swabs and (b) whole blood of horses tested for African horse sickness virus in Eastern and Central Saudi Arabia, 2014–2016

The control positive curve begins early in the cycle (Ct=17)



Lane C: positive control

Lane 1: negative control

Lanes 2-13: nasal swabs

Lanes 16-27: rectal swabs

Lanes 2 and 16: non-template, non-primer lanes containing pure water

Lane M: DNA ladder

Fig. 3

An amplification gel of the partial AHSV-NS2 gene achieved using gel electrophoresis on reverse transcriptase-polymerase chain reaction products from selected swabs from Eastern and Central Saudi Arabia horses tested for African horse sickness virus, 2014–2016