

Detection of African horsesickness virus and discrimination between two equine orbivirus serogroups by reverse transcription polymerase chain reaction

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ABSTRACT

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A reverse transcription polymerase chain reaction (RT-PCR), based on the gene encoding the NS2 protein of African horsesickness virus (AHSV), was developed for rapid serogroup-specific detection of AHSV. The specificity of RT-PCR products was confirmed by Southern blot hybridization using a radioactively labelled cDNA probe specific for the NS2 gene. This RT-PCR could discriminate between all known members of the AHSV and equine encephalosis virus serogroups. AHSV RNA was detected in a sample representing 0,005 plaque forming units in a dilution series made of infected cell culture material. In an immune horse which had been vaccinated with a baculovirus expressed AHSV (serotype 4) VP2 subunit vaccine, viral RNA could be detected for up to 22 weeks post challenge. AHSV RNA was detected in various organs of an infected horse. Viral RNA was also detected by RT-PCR in nine suspected field cases of African horsesickness while virus isolation was successfully performed on eight of these cases.

Keywords: African horsesickness virus, equine encephalosis virus, reverse transcription polymerase chain reaction

INTRODUCTION

African horsesickness (AHS) is enzootic in sub-Saharan Africa. The disease has a high mortality rate (70–95 %) (Coetzer & Erasmus 1994a) and when it occurs in areas outside Africa it usually results in many losses (McIntosh 1958). The aetiological agent is African horsesickness virus (AHSV), a member of the *Orbivirus* genus of the family Reoviridae. The virus contains a segmented dsRNA genome consisting of ten genes which encode seven structural and four non-structural proteins (Oellermann, Els & Erasmus 1970; Bremer 1976; Huismans & Els 1979; Devaney, Kendall & Grubman 1988; Van Staden, Theron, Greyling, Huismans & Nel 1991; Van Staden & Huismans 1991; Grubman & Lewis 1992; Laviada, Arias & Sánchez-Vizcaíno 1993).

In an outbreak of AHS the importance of rapid and specific detection of AHSV cannot be overestimated. The classical techniques of virus detection such as virus isolation or intracerebral injection of infant mice, take time and may delay the rapid implementation of control measures. The use of dot blot and northern blot hybridization has been described (Bremer, Huismans & van Dijk 1990), but these methods are generally not sensitive enough for the detection of AHSV RNA from field samples. Various enzyme linked immunosorbent assays (ELISAs) have been described for the detection of AHSV (Du Plessis, Van Wyngaardt & Bremer 1990; Du Plessis, Van Wyngaardt, Gerdes & Opperman 1991; Hamblin, Mertens & Boned 1991; Hamblin, Mertens, Mellor, Burroughs & Crowther 1991; Hamblin, Anderson, Mellor, Graham, Mertens & Burroughs 1992; Ranz, Miguët, Anaya, Venteo, Cortes, Vela & Sanz 1992; Laviada, Babin, Dominguez & Sánchez-Vizcaíno 1992).

Although rapid, some of these methods lack the sensitivity to detect viral antigen in all samples containing detectable amounts of virus.

In South Africa, members of another *Orbivirus* serogroup infecting *Equidae*, namely the equine encephalosis virus (EEV) serogroup, can create a diagnostic problem in the case of an outbreak of AHSV, since initial clinical signs of the two diseases are often confused. EEV causes a disease characterized by a high morbidity (60–70 %) but a low mortality (5 %) (Coetzer & Erasmus 1994b). Rapid discrimination between AHS and equine encephalosis is of the utmost importance for the introduction of suitable control measures.

Recently it was shown that horses immunized with subunit vaccines consisting of the outer capsid protein VP2 of AHSV serotype 4 (AHSV4), or of VP2 in combination with two other structural proteins VP5 and VP7, were protected against challenge with a virulent dose of AHSV4 (Martinez-Torrecuadrada, Diaz-Laviada, Roy, Sanchez, Vela, Sánchez-Vizcaíno & Casal 1996; Stone-Marschat, Moss, Burrage, Barber, Roy & Laegreid 1996; Roy, Bishop, Howard, Aitchison & Erasmus 1996). The use of such subunit vaccines, in combination with a rapid sensitive test for virus detection to confirm the absence of replicating virus, could also benefit the international transport of equines.

The use of the reverse transcription polymerase chain reaction (RT-PCR) could address the above mentioned problem of discriminating between the two orbivirus serogroups. Various RT-PCRs have been developed for the detection of the AHSV serogroup (Zientara, Sailleau, Moulay, Plateau & Cruciere 1993; Sakamoto, Punyahotra, Mizukoshi, Ueda, Imagawa, Sugiura, Kamada & Fukusho 1994; Stone-Marschat, Carville, Skowronek & Laegreid 1994; Zientara, Sailleau, Moulay & Cruciere 1994; Bremer & Viljoen 1995). Differentiation of the group of serotypes comprising AHSV1, AHSV3, AHSV6 and AHSV8 and different serotypes AHSV2, AHSV4, AHSV5, AHSV7 and AHSV9 was also possible by restriction length polymorphism analysis of the group-specific RT-PCR product (Zientara *et al.* 1993).

This paper reports on the application of an AHSV group-specific RT-PCR based on the NS2 gene (Bremer & Viljoen 1995) for the detection of AHSV RNA in infected cell cultures and in the blood and organs of infected horses. The RT and PCR reactions were carried out sequentially in a single tube. The sensitivity of the RT-PCR in infected cell cultures was determined.

MATERIALS AND METHODS

Cells and virus

All virus stocks were obtained from Dr B.J. Erasmus of Onderstepoort Biological Products. AHSV stocks

consisted of cell cultures infected with virulent strains of AHSV serotypes that had been passaged two or three times in mouse brain, plaque purified twice in VERO cell cultures and then passaged once or twice in BHK or CER cell cultures. The EEV serotypes were plaque purified once or twice in VERO cell cultures and cultivated in CER or VERO cell cultures.

Virus isolation

Virus was usually isolated from horse blood by seeding monolayer VERO cell cultures with a 10 % (w/v) suspension of blood in Eagle's medium or a 10 % suspension of macerated organs in buffered lactose peptone. After an incubation period of 15 min, the cells were washed three to four times in Eagle's medium and were then grown in Eagle's medium. The medium as well as the buffered lactose peptone contained appropriate antibiotics and Fungizone [amphotericin (E.R. Squibb & Sons Inc., Princeton, NJ)]. Cells were monitored daily for cytopathic changes. Infant mice were inoculated intracerebrally with appropriate volumes of the same suspensions and were observed daily for clinical signs.

Experimental animal

A horse was injected twice with a lysate containing AHSV4 VP2 (produced by recombinant baculoviruses in *Spodoptera frugiperda* cells) before challenge with infective horse blood containing 10^5 TCID₅₀ AHSV4 ($0,69 \times 10^5$ plaque forming units (PFU) (Roy *et al.* 1996).

Samples

Blood samples were collected in heparinized tubes from the jugular vein. In general, 1 ml volumes were transferred to Eppendorf tubes and plasma was separated from the blood cells by low speed centrifugation ($500 \times g$) and removed. RNA was then extracted directly from the packed blood cells. Alternatively, blood cells were stored at -20°C or -70°C before RNA was extracted. Samples from organs were stored at either 4°C or -20°C before RNA extraction.

RNA extraction

The acid-phenol-chloroform method (Chomczynski & Sacchi 1987) was used for the extraction of total RNA from infected CER cell cultures and from blood and organs. For RNA extraction from samples obtained from horses, a suspension was usually made of approximately 300 μg of organ material or of the blood cell pellet contained in 1 ml blood, in 500 μl solution D (4 M guanidium thiocyanate, 25 mM sodium citrate, pH 7, 0,5 % sarkosyl and 0,1 M β -mercapto ethanol). Thereafter, 50 μl of 2 M sodium acetate (pH 4), 500 μl water saturated phenol and 100 μl chloroform:isoamyl alcohol (49:1) were added.

After centrifugation (18 000 $\times g$ for 15 min), the RNA in the supernatant fluid was precipitated with isopropanol. In certain instances 10–20 μg glycogen was added. The pellets were washed in 75 % ethanol and resuspended in distilled pyrogen-free water. In some instances a second extraction was carried out on samples to ensure that all inhibitory substances were removed.

Primer design

Primers based on sequences contained in conserved regions of the AHSV NS2 gene (Bremer *et al.* 1990) as previously described (Bremer & Viljoen 1995) consisted of the following sequences:

5'-TGGCACGAAGACATG (position 699–713)

and

5'-CTCTCATCGCCAATG (position 929–915).

These were used for both cDNA synthesis and subsequent PCR.

RT-PCR analysis

Total extracted RNA resuspended in distilled pyrogen-free water, was added to 50 ng of each primer and the volume was adjusted to 5 μl with distilled pyrogen-free water. The sample was heated for 3 min at 96 °C and cooled rapidly on ice. A 5 μl volume of a mixture containing 10 U M-MLV reverse transcriptase (RT) (Promega), 2 \times M-MLV buffer and 10 mM of each dNTP and approximately 25 U human placental RNase inhibitor (Amersham) was added. Samples were incubated at 37 °C for 45 min. Some samples were incubated at 95 °C for 5 min to denature the RT (Sellner, Coelen & MacKenzie 1992).

PCR (Saiki, Scharf, Faloona, Mullis, Horn, Erlich & Arnheim 1985) was carried out by adding 40 μl of a mixture containing 2 U thermostable DNA polymerase (DynazymeTM, Finnzymes), 1,2 \times Dynazyme buffer and 200 μM of each dNTP to each sample. The reaction was carried out in a Perkin Elmer 9600 PCR machine using the following conditions: 96 °C for 3 min, followed by 40 cycles of 96 °C for 20 s, 57 °C for 30 s and 72 °C for 30 s. The RT-PCR amplicons were analyzed by agarose gel electrophoresis.

Southern blot hybridization

The amplified DNA products were analyzed by electrophoresis on 1,5 % agarose gels and were visualized by ethidium bromide staining and UV irradiation. Southern blot analysis was carried out using slightly modified standard procedures (Ausubel, Brent, Kingston, Moore, Seidman, Smith & Struhl 1987). DNA was transferred to a Zeta-Probe membrane in 0,4 M NaOH using a RED-EVAC vacuum blot apparatus (Hofer, San Francisco). The membrane was washed in 2 \times SSC (0,3 M NaCl, 0,03 M sodium citrate,

pH 7) and exposed to microwaves for 2,5 min (850 W). Prehybridization was carried out in 50 % formamide, 5 \times SSC, 5 \times Denhardt's solution, 0,05 M sodium phosphate buffer pH 6,8, 1 % sodium dodecyl sulphate (SDS) and 0,25 mg/ml herring or salmon sperm DNA at 42 °C for 1–16 h and hybridization was carried out at the same temperature in 50 % formamide, 4 \times Denhardt's solution, 4 \times SSC, 0,05 M sodium phosphate buffer pH 6,8 and 10 % dextran sulphate, 0,25 mg/ml salmon or herring sperm DNA. Blots were washed 4 \times 15 min in 0,1 \times SSC and 0,1 % SDS at 65 °C. The probe used consisted of the GC-tailed full length AHSV3 NS2 gene excised from the *Pst* 1 site of the plasmid Bluescribe into which the gene had been ligated after it had been excised from the original clone (Bremer *et al.* 1990). The NS2 gene was separated from the plasmid by agarose gel electrophoresis, purified and labelled with ³²P [Multi-primeTM random priming labelling system (Amersham)]. The labelled probes on average contained 0,5–4 $\times 10^7$ cpm ³²P.

RESULTS

Serogroup reactivity

To determine whether the amplification strategy could differentiate between the AHSV and EEV serogroups, total RNA was extracted from virus infected or uninfected CER cells and the respective RNA samples were then subjected to RT-PCR. Amplicons were analyzed by agarose gel electrophoresis (Fig. 1A) and Southern blot hybridization (Fig. 1B) as described above.

A 230 bp amplicon was obtained from RNA extracted from all nine AHSV serotypes respectively, but not from RNA extracted from the seven EEV serotypes or the negative control samples. Additional bands (smaller or larger than 230 bp) were observed in some samples (e.g. Fig. 1A: CER control and EEV serotype Bryanston), but these were shown to be non-specific by Southern blot hybridization (Fig. 1B). An EEV serogroup specific RT-PCR has not been developed and the presence of EEV RNA in the different samples was verified by agarose gel electrophoresis. A typical electrophoretic profile, similar to that obtained by Viljoen & Huismans (1989) representing the ten dsRNA segments, was identified for each serotype (result not shown). In order to ensure that the lack of RT-PCR products in the different EEV samples was not due to the presence of inhibiting substances, duplicate RNA samples were prepared of each serotype. To one of each, a sample consisting of 2 ng total RNA extracted from AHSV3 infected cells, was added as internal control. RT-PCR was carried out and a product of 230 bp was observed in each each sample containing AHSV, indicating that the RT-PCR conditions were appropriate. In both the

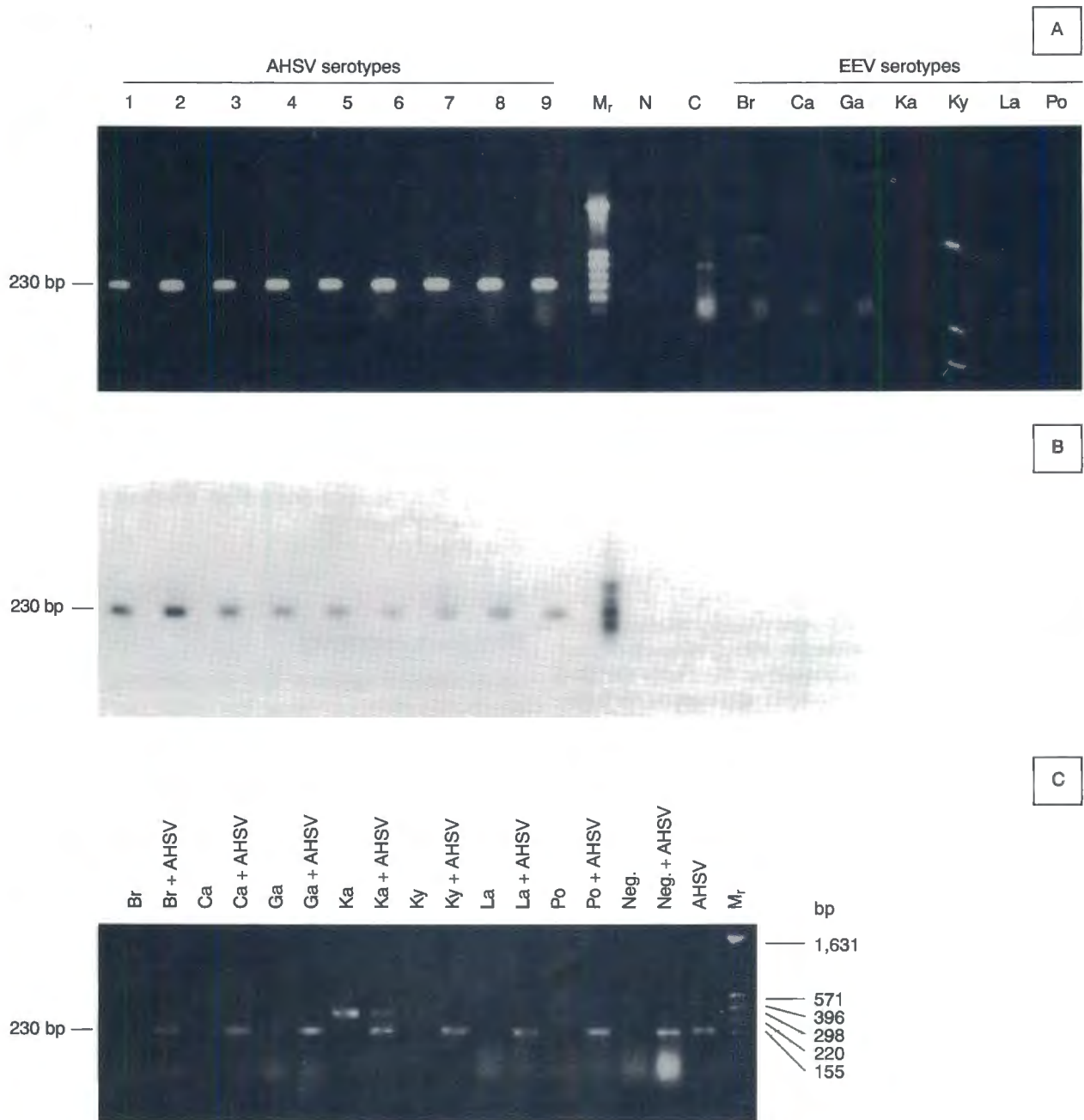


FIG. 1 Specificity of the RT-PCR. RT-PCR was performed on RNA extracted from CER cells infected with the nine different AHSV serotypes as well as the seven EEV serotypes respectively, as well as from uninfected cells. Products were analyzed by electrophoresis in an ethidium bromide stained 1,5 % agarose gel (A). DNA was transferred to Zeta-Probe membranes and analyzed by Southern blot hybridization using a ³²P-labelled NS2 gene specific probe. The autoradiogram (B) indicates the results

Samples in (A) and (B) are RT-PCR products obtained from total RNA extracted from AHSV serotypes 1–9 as indicated, uninfected CER cells (C), and EEV serotypes Bryanston (Br), Cascara (Ca), Gamil (Ga), Kaalplaas (Ka), Kyalami (Ky), Langeberg (La), Potchefstroom (Po). A control in which the template is replaced by water (N) is also included. M_r is the DNA size marker consisting of ³²P-labelled *Hinf* 1 digested pAT153

Fig. 1C indicates RT-PCR products obtained from duplicate samples of RNA extracted from uninfected CER cells, or from CER cells infected with the different EEV serotypes in the absence (Br, Ca, Ga, Ka, Ky, La, Po, Neg.) or presence of AHSV RNA (Br + AHSV, Ca + AHSV, Ga + AHSV, Ka + AHSV, Ky + AHSV, La + AHSV, Po + AHSV, Neg. + AHSV) as indicated. Products were analyzed as described in (A). M_r comprises *Hinf* 1 digested pAT153. The sizes of DNA fragments are indicated on the right

Kaalplaas samples a replicon of approximately 400 bp was observed. A faint band of the same size was also present on the gel shown in Fig. 1A, although not clearly visible in the photograph. Southern blot hybridization indicated that the product was not AHSV specific (Fig. 1B).

Sensitivity of RT-PCR

The sensitivity of the RT-PCR was determined by extracting RNA from samples taken from a dilution series made of AHSV3 infected CER cell culture material containing 2×10^5 PFU. RNA was extracted from $100 \mu\ell$ of each dilution, and RNA contained in $1/4$ of the purified product was analyzed by RT-PCR. RT-PCR was carried out on each sample and the amplified DNA products were analyzed by agarose gel electrophoresis and Southern blot hybridization. The lowest dilution at which a faint band was visible on an ethidium bromide stained agarose gel was from a suspension containing $0,2$ PFU/ml, representative of $0,005$ PFU in total. The specificity of the DNA product was indicated by Southern blot hybridization (Fig. 2).

Detection of viral RNA in infected horses

Experimentally infected horse

Although challenge with a lethal dose of virus does not exactly simulate natural infection taking place in a field situation, it was of interest to determine the interval during which AHSV RNA could be detected in a challenged horse that had been protected by immunization with a subunit vaccine. In short, a horse was injected with approximately $5 \mu\text{g}$ of AHSV4 VP2 (produced by recombinant baculoviruses) and challenged with virulent AHSV4 as described (Roy *et al.* 1996). Blood samples were collected at regular intervals and no virus could be isolated from blood samples for a period of 12 d post challenge. RT-PCR was carried out on total RNA extracted from blood samples taken at weeks 3, 4, 6, 10, 14, 18, 22, 26 and 30 as described in Materials and methods. RT-PCR RNA was usually extracted shortly after the sample had been taken and RT-PCR was carried out to determine whether AHSV RNA could be detected. After Southern blot hybridization, a positive signal was obtained in samples taken at weeks 3, 4, 6, 10, 14, 18 and 22. RNA samples were stored at -20°C . After investigating the sample taken at 30 weeks post challenge, RT-PCR was again carried out simultaneously on samples taken at weeks 18, 22, 26 and 30. RT-PCR amplicons were analyzed by agarose gel electrophoresis followed by Southern blot hybridization. AHSV RNA could be detected up to at least 22 weeks post challenge (Fig. 3). It is not clear why the signal obtained from the blood sample taken at 18 weeks post infection was weaker than that obtained at week 22.

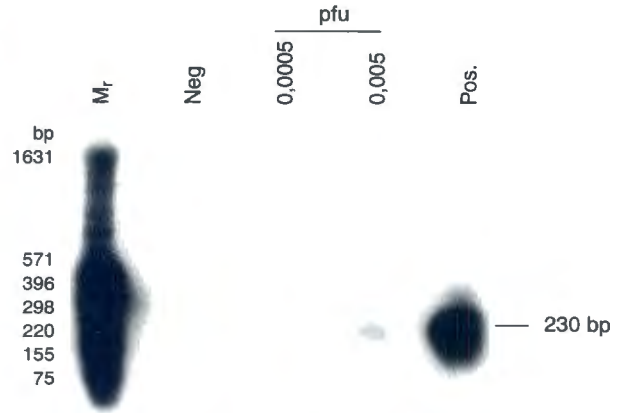


FIG. 2 Analysis of the sensitivity of RT-PCR. RT-PCR was carried out on samples of RNA extracted from a dilution series of an AHSV infected cell culture containing 2×10^5 PFU/ml. Analysis was performed as described in Fig. 1. The autoradiogram contains RT-PCR products representing $0,0005$ PFU and $0,005$ PFU, AHSV3 RNA positive control, ^{32}P -labelled *Hinf* 1 digested pAT153 (sizes of fragments indicated on the left of the figure) and a negative control in which water replaced the template (Neg.).

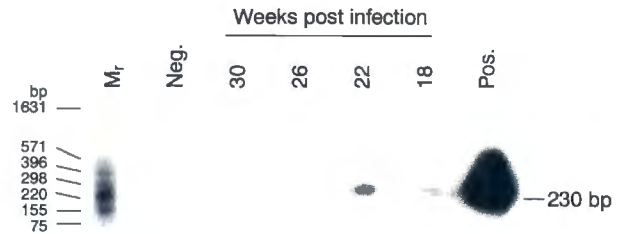


FIG. 3 RT-PCR detection of AHSV RNA in a horse which had been challenged with a lethal dose of AHSV serotype 4, after immunization with baculovirus recombinant VP2 subunit vaccine. Samples were collected at different intervals after challenge. RNA was extracted and subjected to RT-PCR. Analysis of products was performed as described in Fig. 1. Samples represented in the autoradiogram are ^{32}P labelled *Hinf* 1 digested pAT153 (M_r) (sizes of fragments are indicated on the left), RT-PCR products of a control in which water replaced the template (Neg.), RNA extracted from blood taken at 18, 22, 26 and 30 weeks after challenge respectively, and of total RNA extracted from AHSV3 infected CER cells (Pos.).

Field cases

Spleen or lung samples of nine suspected AHS field cases were analyzed for the presence of viral RNA. In seven unvaccinated horses and in one horse vaccinated 2 weeks before death, virus was detected by both virus isolation and RT-PCR. In horse 31/96, last vaccinated in 1994 [using the vaccine strains of serotypes 1, 3 and 4 (26.09.94) followed by the vaccine strain of serotype 5 (27.12.94)], no virus was detected by virus isolation but the presence of viral RNA was shown by RT-PCR. In eight of these cases, RT-PCR products were visible after analysis by agarose

TABLE 1 Comparison of virus isolation and RT-PCR for the detection of AHSV

Date of sample	Number	Date of last vaccination	Virus isolation ^a	RT-PCR
08.02.96	7/96	23.01.96	+	+
08.02.96	8/96	Not vaccinated	+	+
16.02.96	16/96	Not vaccinated	+	+
18.02.96	21/96	Not vaccinated	+	+
22.02.96	24/96	Not vaccinated	+	+
28.02.96	31/96	27.12.94	-	+
14.03.96	49/96	Not vaccinated	+	+
19.03.96	54/96	Not vaccinated	+	+
19.03.96	55/96	Not vaccinated	+	+

^a An organ suspension of each sample was injected intracerebrally into infant mice brain and also used to infect cell cultures

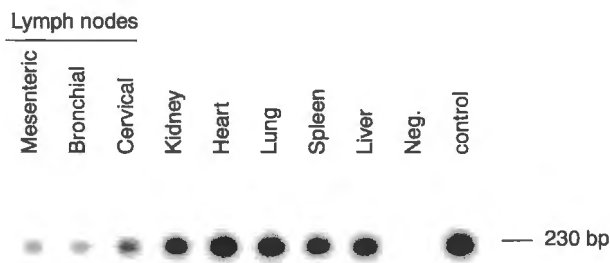


FIG. 4 RT-PCR detection of AHSV RNA in different organs from a necropsy of an infected horse. The RT-PCR products were analyzed as described in Fig. 1. The autoradiogram represents RT-PCR products obtained from RNA extracted from the different organs of a suspected AHS field case as indicated, as well as from the spleen of a horse which had been injected with AHSV (Pos.). In the negative control water was used as template in the RT-PCR (Neg.)

gel electrophoresis. The RT-PCR product obtained from horse 55/96 was only detected after Southern blot hybridization.

Furthermore, we wanted to determine whether AHSV RNA could be detected in other organs of horse 31/96. Viral RNA could be detected in all the organs tested (Fig. 4). The autoradiogram indicated that most amplicons were obtained from RNA extracted from the spleen, lung, liver and heart. Slightly less amplicons were obtained from the kidney and the cervical lymph node and the least amplicons from the bronchial and mesenteric lymph nodes.

DISCUSSION

The rapid specific detection of AHSV by means of RT-PCR would allow the quick introduction of suitable control measures to limit the spread of AHS. Various group-specific RT-PCR procedures based on AHSV genes encoding proteins VP3 (Sakamoto *et al.* 1994), VP7 (Zientara *et al.* 1993; 1994) and NS2 (Bremer & Viljoen 1995; Stone-Marschat *et al.* 1994)

have already been described. We have adapted a previously described NS2 gene-based AHSV RT-PCR (Bremer & Viljoen 1995). The procedure was evaluated to determine its suitability as a routine diagnostic procedure by applying it to samples obtained from infected cell cultures and from infected horses.

The described RT-PCR followed by analysis of products by agarose gel electrophoresis could provide a result within approximately 7 h after initiation of RNA extraction. The specificity of products could be determined by Southern blot hybridization within an additional 24 h. This period of time is significantly shorter than virus isolation which often takes up to 2 weeks.

As a result of the incidence of two different equine orbiviral diseases in southern Africa, all known members of the two different equine orbiviral serogroups had to be tested by RT-PCR. We clearly indicated that the procedure has the potential to be used as a routine diagnostic test for AHSV in samples from regions where both diseases are enzootic, because all of the nine known AHSV serotypes, but none of the seven known EEV serotypes were detected. Previously, RNA of one EEV serotype was included as a control in an AHSV specific RT-PCR (Stone-Marschat *et al.* 1994). This is the first study in which all the different known EEV serotypes were included.

The RT-PCR should detect low amounts of virus in order to be acceptable for the certification of virus-free samples for quarantine purposes. We were able to detect RNA representing as little as 0,005 PFU of virus in infected cell cultures. This is more sensitive than the group-specific AHSV RT-PCR based on the AHSV VP 3 gene, which could detect 10⁰-10¹ TCID₅₀ (Sakamoto *et al.* 1994) without the use of an AHSV specific probe. A RT-PCR in which chemiluminescent label was incorporated into PCR products derived from bluetongue virus (BTV) RNA was described in which 1,6 x 10² PFU/ml of BTV in semen could be detected (Akita, Glenn, Castro & Osburn 1993).

AHSV RNA could be detected by RT-PCR followed by Southern blot hybridization for at least 22 weeks post challenge in a blood sample from a horse immunized with recombinant AHSV VP2. No virus could be isolated from this horse for a period of 12 d post challenge. A fraction of the AHSV particles could be present in the form of non-infectious core-like particles which is detectable by RT-PCR but not by virus isolation. Brewer & MacLachlan (1992) observed that BTV particles associated with ruminant erythrocytes, often resembled viral cores, which lack the outer capsid proteins required for the infection of mammalian cells. This was suggested as the reason for the detection of BTV RNA in blood samples from infected calves by RT-PCR followed by enzyme-linked immunosorbent assay (ELISA) up to 20 weeks

post infection while virus could only be isolated for a period of 2–8 weeks post infection. It was suggested that the detection of BTV RNA was linked to the life span of the bovine erythrocyte (Brewer & MacLachlan 1992; MacLachlan, Nunamaker, Katz, Sawyer, Akita, Osburn & Tabachnick 1994).

The RT-PCR could detect viral RNA in the lung and spleen of horse 31/96 (Table 1) from which no virus could be isolated. The level of neutralizing antibodies induced by vaccination, was probably too low to protect the horse against natural infection after such an extended period (14 months post vaccination), resulting in the death of the horse.

Samples from various organs of horse 31/96 were investigated by RT-PCR as shown in Fig. 4. Virus isolation was attempted only from the lung and the spleen of this horse. Samples from organs other than the lung and spleen could therefore be sent as specimens for laboratory analysis. ELISA results indicated that the antigen concentration was the highest in the spleen, although considerable quantities of virus were contained in the heart, lung and liver (Hamblin *et al.* 1992).

The RT-PCR described in this paper can be used in the routine laboratory diagnosis of AHSV. It also has the potential to be used in the epidemiology of the disease for the detection of virus in reservoirs and vectors of the virus.

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