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Diagnosis and molecular epidemiology of the African horsesickness virus by the polymerase chain reaction and restriction patterns

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Summary — African horsesickness is a viral disease caused by an orbivirus belonging to the Reoviridae family. This paper describes a polymerase chain reaction (PCR) for amplifying segments 7, which encode for VP 7, a protein common to the 9 known serotypes of this virus. A reverse transcription step is necessary before amplification. No amplified product could be observed in cell cultures infected with other equine viruses. The amplified DNAs were digested to completion by 8 different restriction enzymes. The restriction fragment length polymorphisms allowed the differentiation of the group of serotypes AHSV-1, 3, 6, 8 and the viruses AHSV-2, AHSV-4, AHSV-5, AHSV-7 and AHSV-9. Differences could also be described between vaccinal strains of the same serotype produced in cell cultures or in brains of suckling mice.

African horsesickness virus / reverse transcription / polymerase chain reaction / diagnosis

Résumé — Diagnostic et épidémiologie moléculaire du virus de la peste équine par amplification génique et étude des profils de restriction. La peste équine est une maladie virale, affectant les Équidés, due à un orbivirus de la famille des Réoviridae. Cet article décrit l'application de la technique d'amplification du gène 7 qui code pour VP 7, une protéine interne de capsid commune aux 9 sérotypes connus de ce virus. Une étape de transcription inverse est nécessaire avant amplification. Aucun produit d'amplification n'est observé à partir de cultures de cellules inoculées avec d'autres virus pathogènes pour les chevaux. Les ADN amplifiés sont hydrolysés par 8 endonucléases de restriction. Les profils de restriction permettent de regrouper les sérotypes 1, 3, 6, et 8 et de différencier entre eux les sérotypes 2, 4, 5, 7 et 9. Des différences peuvent être observées entre les profils des souches vaccinales du même sérotype mais produites sur cellules ou sur cerveaux de souriceaux.

peste équine / transcription inverse / amplification de gènes / diagnostic

INTRODUCTION

African horsesickness is a viral disease of the Equidae caused by an orbivirus belonging to the Reoviridae family (Verwoerd *et al*, 1979) and very close genetically and structurally to the bluetongue virus. The virus is transmitted by biting insects (Culicoides) (Du Toit, 1944) which are biological vectors. Nine serotypes of the virus have been described (Mc Intosh, 1958). In July 1987, Spain became infected after the importation of zebras (which are less sensitive than horses to the virus) from Namibia. The disease has subsequently spread to Portugal and Morocco.

The African horsesickness virus (AHSV) genome is composed of 10 double-stranded RNA molecules (Oellermann *et al*, 1970; Bremer, 1976). The virion consists of 7 structural proteins, and a variety of non-structural proteins are also synthesized in AHSV-infected cells.

Recent analysis of the coding assignment for AHSV-4 strain genes has revealed that segments 1, 2, 3 and 4 respectively encode VP1, VP2, VP3 and VP4, segment 5 encodes NS1, segment 6 encodes VP5 and VP6, segment 7 encodes VP7, segment 9 encodes NS3 and segment 10 encodes NS4 and NS4a (Grubman and Lewis, 1992; Mizukoshi *et al*, 1992).

The outer capsid is composed of the 2 major proteins (VP2 and VP5) which are responsible for the viral neutralization and antigenic variability, whereas the inner capsid is composed of 2 major (VP3 and VP7) and 3 minor (VP1, VP4 and VP6) proteins. VP7 is common for all 9 serotypes (Bremer *et al*, 1990; Chuma *et al*, 1992) and is involved in the complement fixation test. The classical methods of direct diagnosis of this infection are intracerebral inoculation in suckling mice or infection of cell cultures (BHK21 or Vero

cells). The serological diagnosis is based on the complement fixation test which is the official method for international trade of horses. But for all these techniques the time between the awareness of a suspected sickness and the response of the (veterinary) laboratory is always considered by the veterinary authorities as being too long (due to the poor conditions of conservation of the samples, the time necessary to serotype the virus, etc). That is why new methods of diagnosis have been studied, particularly based on the molecular biology of the virus. We have developed a polymerase chain reaction (PCR) analysis to rapidly identify and serotype the African horse sickness disease virus.

MATERIALS AND METHODS

Viruses and cells

African horsesickness strains

The vaccine strains of the 9 AHSV serotypes were kindly provided by Dr Pearson (US Dept of Agriculture, National Veterinary Services Laboratories, Ames, IA). All strain serotypes were obtained from South Africa (Dr Erasmus, Onderstepoort Veterinary Research Institute, South Africa) except the strain serotypes 8 and 9, which were isolated in Iran. Viruses had been prepared in 1968 and 1969 by passaging each virus 100 times in suckling mice *via* intracranial inoculation. Commercially available vaccinal strains AHSV-9 and AHSV-4 strains (Onderstepoort Veterinary Research Institute, South Africa) were also used in this study.

All AHSV viruses were propagated on Vero (ref American type culture collection [ATCC] CCI 81) cell lines in 175-cm² flasks in growth medium RPMI 1640 supplemented with 8% foetal calf serum, streptomycin (100 µg/ml) and penicillin (100 IU/ml). The infected monolayers showed cytopathic effects from 48 h to 96 h later depending on the strains. The titres, as determined by the Reed and Muench (1938) method of estimating 50% end-points, varied from 10⁵ to 10⁷ TCID₅₀/ml.

Other equine viral strains

The equine arteritis virus (EAV), and reovirus serotype 1 were propagated on Vero cell lines, the equine abortion virus (EHV1) Kentucky strain on RK13 cells (ATCC CCI 106), the influenza A/equi 2/Brentwood 79 virus on MDCK cells (ATCC CCI 34), and the equine adenovirus 1 on equine dermis cells (ATCC CCI 57). All infected and non infected cell cultures were treated by the same procedure.

Nucleic acid sample preparation

Extractions of total RNA were performed on infected and uninfected cell cultures. Cell debris was removed by low-speed centrifugation: 10 min at 500 *g*. The supernatants were ultracentrifuged at 100 000 *g* for 3 h at 4°C in a SW28-rotor (Beckmann rotor). As already described by Spaan *et al* (1981), the pellets were resuspended in 200 µl TNE (Tris 10 mM, pH 7.5, NaCl 100 mM, EDTA 1 mM) for each serotype and were treated with proteinase K (0.2 mg/ml) for 30 min at 37°C, solubilized by the addition of 180 µl TNE 2X and 20 µl sodium-dodecyl sulfate (SDS 20%) (50 min at 50°C and 30 min at 25°C), and sequentially extracted by phenol-chloroform as described for large-scale DNA preparation (Sambrook *et al*, 1989). The nucleic acids were precipitated from the aqueous phase in the presence of sodium acetate and ethanol, dried (under vacuum) and resuspended in 10 µl diethylpyrocarbonate (DEPC)-treated water. The same protocol was used for all nucleic acid extractions.

Primers

Sequence data and cross-hybridization experiments have indicated that all orbiviruses have common and characteristic 5' and 3'RNA segment terminal sequences, namely 5' GTTAAA 3' and 5' ACTTAC 3' (Rao *et al*, 1983; Mertens and Sangar, 1985).

The selection of oligonucleotide primer sequences used in the PCR protocol was determined from sequence data published on AHSV-4 segment 7 gene (a strain isolated in Spain in 1987), which codes for AHSV peptide VP 7 (Roy

et al, 1991). This segment is 1179 bp long and the M_r value of the ds RNA is calculated to be 7.7×10^5 Da. Nucleotides 1 to 20 and 1159 to 1179 were chosen as upstream and downstream respectively. The sequences of the 2 primers were as follows: 5'G T T A A A T T C G G T T A G G A T G 3' for the upstream primer and 5' G T A A G T G T A T T C G G T A T T G A 3' for the downstream primer. The oligonucleotides were synthesized at 0.2 µmol in a Gene Assembler Plus (Eurogentec, Seraing, Belgium).

Reverse transcription and polymerase chain reaction

One µg total nucleic acids resuspended in 2.5 µl sterile DEPC-treated water (as previously described) was denatured with an equal volume of 0.02 M methyl mercuric hydroxide (Wade-Evans *et al*, 1990) prior to use as a template for cDNA synthesis (10 min at room temperature). The protocol used for the amplification of RNA template was an adaptation of previously published protocols (Doherty *et al*, 1989; Wade-Evans *et al*, 1990).

After reduction of the methyl mercuric hydroxide by addition of 1 µl 0.7 M-β-mercaptoethanol, 2 µl RNAsin (40 units/µl; Boehringer-Mannheim, Meylan, France) were added (5 min at room temperature). Four µl of the mix were diluted to 20 µl in 50 mM Tris pH 8.3, 40 mM KCl, 1 mM DTT, 1 mM of each dNTP, 6 mM MgCl₂ containing 10 pmol of the pair of primers. Twenty units of avian myeloblastosis virus reverse transcriptase (AMV-RT; Appligene, Illkirch, France) were added to the reaction mix which was incubated at 37°C for 1 h. One µl cDNA reaction mix was diluted to 100 µl for a final concentration of 10 mM Tris, pH 8.8, 1.5 mM MgCl₂, 50 mM KCl, 200 µM of each dNTP. Five pmol of each primer and 2.5 units Taq DNA polymerase (Boehringer-Mannheim) were added prior to incubation on a thermocycler PTC 100/60 (Prolabo). A 200-µl mineral oil overlay was applied to each reaction mixture prior to amplification. The mix was heated to 95°C for 5 min, and incubated on the heating block for 40 cycles of 55°C for 1 min, 70°C for 2 min and 95°C for 1 min followed by a terminal extension step at 70°C for 8 min (Saiki *et al*, 1985, 1988). Ten µl amplified sample from each

reaction mixture were analysed in 2% agarose gel (1% Nu-sieve/1% Sea-Kem) (Nalgene, Tebu) in Tris-borate-EDTA buffer 1X, run at 100 V for 2 h, stained with ethidium bromide for 10 min, and visualized by ultraviolet transillumination. Viral amplified AHSV bands were compared to the migration of a pBR328 / *Bgl*I + pBR328/*Hin*II molecular weight standard (Boehringer-Mannheim - cat No 1062590).

Analysis by restriction endonucleases

All the restriction enzymes used to analyse the amplified cDNA products of the 9 serotypes were selected according to the restriction sites of AHSV-4 segment 7 cDNA (Roy *et al*, 1991) (table I). Ten- μ l aliquots of the amplified PCR mixtures from each AHSV serotype 1 to 9 were digested by the restriction enzymes (10 units per reaction) *Asn*I, *Bam*HI, *Hin*II, *Pvu*II, *Sac*I, *Sph*I, *Cfo*I (Boehringer-Mannheim) and *Hph*I (Ozyme) in a total volume of 20 μ l (with the buffers recommended by the manufacturer) at 37°C for 2 h.

*Pvu*II is the single enzyme for which no restriction site could be detected in the genomic

sequence of the AHSV-4 Spanish isolate. The resulting cleavage fragments along with pBR 328/*Bgl*I + pBR328/*Hin*II-DNA standards were separated by electrophoresis through a 2% agarose horizontal slab gel (100 V for 1 h) and visualized as previously described.

RESULTS

Amplification of total extracted RNA from infected and non infected cell cultures

With AHSV viruses

For AHSV serotype 4 (USDA) an amplified product was obtained with a size of 1 179 bp (fig 1, lane 4). Amplified products also appeared as fragments of similar molecular weight for the other AHSV serotypes (fig 1, lanes 1 to 3 and 5 to 9). Figures 2E and 2K show 2 fragments with the same molecular weight for AHSV-4 (South Afri-

Table I. Restriction enzymes used to analyse the PCR amplified products and indication of their restriction sites on the AHSV-4 segment 7 (Spanish isolate) (Roy *et al*, 1991).

<i>Restriction enzymes (restriction site)</i>	<i>No of sites predicted on segment 7 AHSV-4</i>	<i>Size (in bp) of cleavage fragments</i>
<i>Asn</i> I (ATTAAT)	3	432; 348; 273; 126
<i>Bam</i> HI (GGATCC)	1	1 087; 92
<i>Hin</i> II (GANTC)	1	1 030; 149
<i>Hph</i> I (GGTGA)	3	444; 397; 258; 80
<i>Pvu</i> II (CAGCTG)	0	—
<i>Sac</i> I (GAGCTC)	2	759; 292; 128
<i>Sph</i> I (GCATGC)	1	1122; 57
<i>Cfo</i> I (GCGC)	5	511; 243; 158; 150; 97; 20

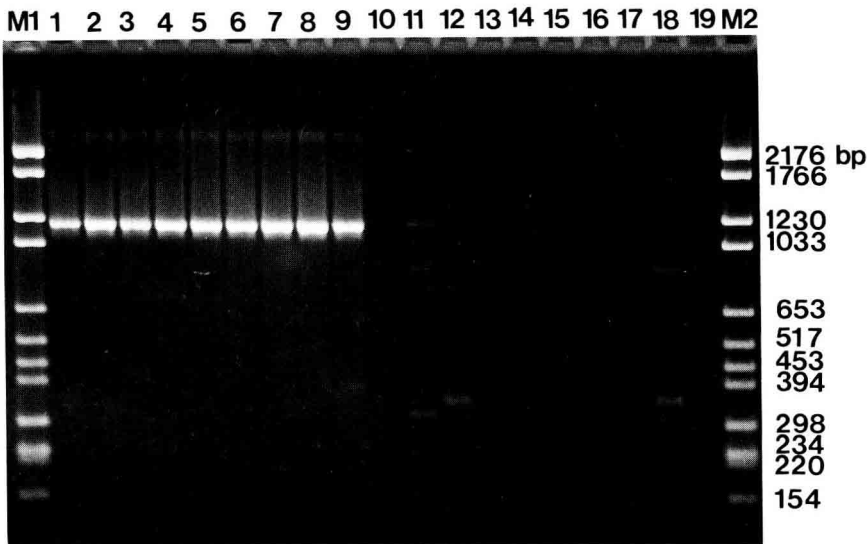


Fig 1. Amplification of AHSV ds RNA segments 7 template. Lanes M1 and M2: pBR 328/*Bgl*I + pBR328/*Hinf*I-DNA marker. Lanes 1 to 9: PCR amplification products of the 9 AHSV serotypes ds RNA from infected Vero cell cultures (lane 1: AHSV-1, lane 2: AHSV-2, ..., lane 9: AHSV-9). Tracks 10, 11, 13, 15, 17 contain samples of PCR using total nucleic acid extracted from cell cultures infected with: 10) equine arteritis virus; 11) reovirus 1; 13) equine abortion virus EHV 1; 15) influenza A/equi 2/Brentwood 79; 17) equine adenovirus 1. Tracks 12, 14, 16, 18: PCR using total nucleic acid extracted from: 12) Vero cells; 14) RK 13 cells; 16) MDCK cells; 18) equine dermis cells. Lane 19: PCR water control.

ca) (fig 2E, lane 9) and AHSV-9 strains (South Africa) (fig 2K, lane 9). No amplification products were present in samples containing only sterile water (fig 1, lane 19), or in samples prepared from mock-infected cell cultures (fig 1, lane 12). No amplified fragments could be detected from the non-infected equine dermis cell cultures, indicating that there was no amplification of any equine cellular genomic DNA fragment (fig 1, lane 18).

With other equine viruses

As seen in figure 1, no amplified fragments were noted in lanes containing amplification reactions of total nucleic acid extract-

ed from infected Vero cells with respectively equine arteritis virus, reovirus serotype 1 (lanes 10, 11), EHV-1-infected RK13 cells (lane 13), MDCK cells infected with the influenza virus A/equi 2/Brentwood 79 and equine dermis cells infected with equine adenovirus 1 (lanes 15, 17). No cDNA fragment could be detected in the non-infected cell cultures.

Restriction endonuclease DNA fingerprints of the different AHSV fragments 7 cDNA

Figure 2 presents the electrophoresis of the cleavage fragments 7 with the 8 restric-

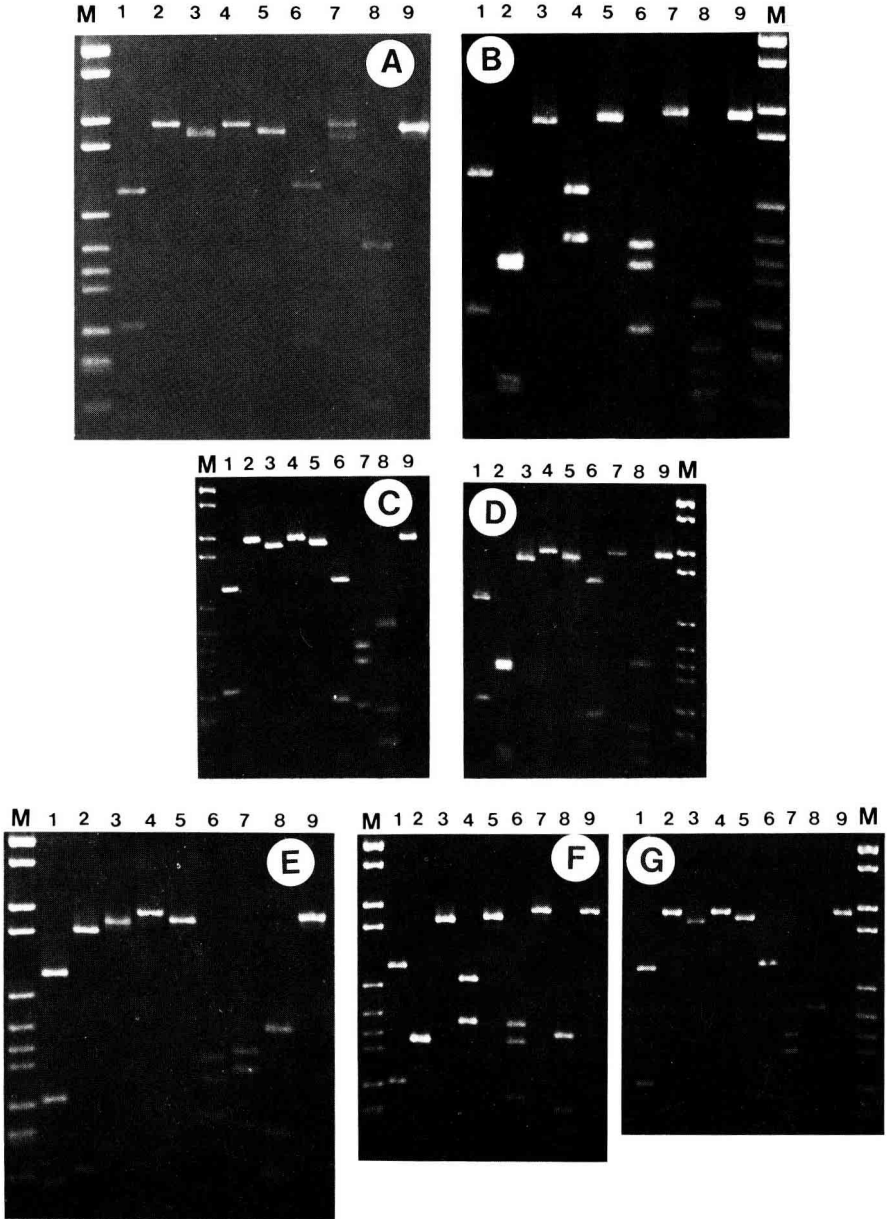
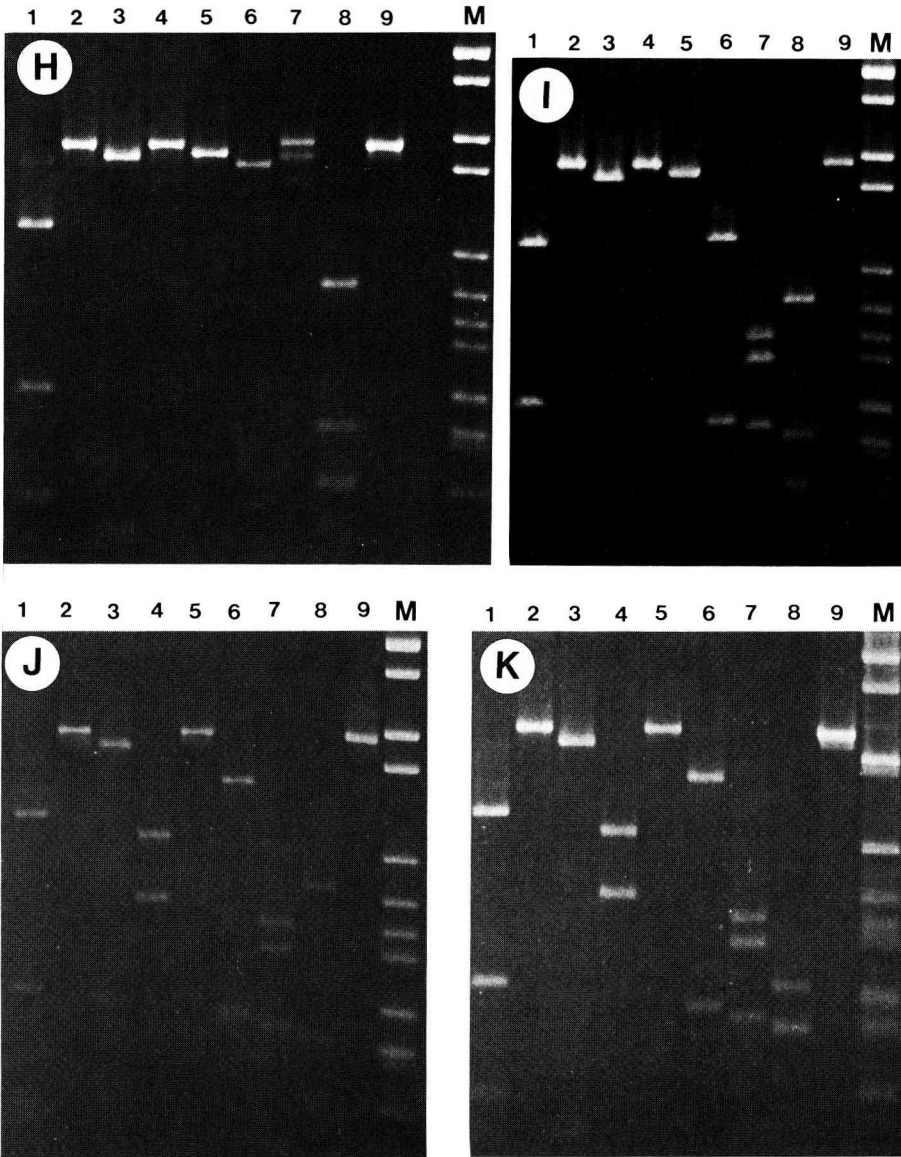


Fig 2. Restriction patterns of the PCR amplified segments 7 genes of each of the 9 AHSV serotypes: A: AHSV-1 (USDA); B: AHSV-2 (USDA); C: AHSV-3 (USDA); D: AHSV-4 (USDA); E: AHSV-4 (South Africa); F: AHSV-5 (USDA); G: AHSV-6 (USDA); H: AHSV-7 (USDA); I: AHSV-8 (USDA); J: AHSV-9 (USDA); K: AHSV-9 (South Africa) digested by restriction enzymes. Lane 1: *SacI*; lane 2: *HinI*; lane 3: *BamHI*; lane 4: *PvuII*; lane 5: *SphI*; lane 6: *AsnI*; lane 7: *HphI* and lane 8: *CfoI*. Lane 9: non digested PCR amplified AHSV cDNA. M: pBR328/*BglI* + pBR328/*HinI*-DNA molecular weight marker.



tion enzymes. Table II summarizes the restriction patterns of the segments 7 cDNA of the 9 serotypes.

As predicted, the 8 restriction patterns of AHSV-4 (South Africa) strain are identical to those theoretically expected by the

published nucleotide sequence of the segment 7 of the AHSV-4 Spanish isolate (Roy *et al*, 1991). For each enzyme, the ability of digestion has been checked simultaneously with standard DNA which have known restriction sequences (data

Table II. Differentiation of the 9 serotypes according to the restriction fragment length polymorphisms of their segment 7 cDNA obtained with 8 restriction enzymes.

<i>Restriction enzymes</i>	<i>Serotypes with same restriction patterns</i>	<i>Serotypes with specific patterns</i>
<i>AsnI</i>	1, 3, 6, 8 2, 5 4 (USDA), 9 (USDA and S Africa)	7 4 (S Africa)
<i>BamHI</i>	The 9 serotypes have the same profiles	...
<i>HinfI</i>	1, 3, 6, 7, 8, 9 (USDA and S Africa): no sites 4 (USDA), 2, 5	4 (S Africa)
<i>HphI</i>	1, 3, 4 (S Africa), 6, 8, 9 (USDA and S Africa) 2, 4 (USDA), 5: no sites	7
<i>PvuII</i>	1, 3, 4 (USDA and S Africa), 6, 7, 8 2, 5, 9 (USDA and S Africa)	...
<i>SacI</i>	The 9 serotypes have the same profiles	...
<i>SphI</i>	1, 2, 3, 4, 5, 6, 7, 8	9 (USDA and S Africa): no site
<i>CfoI</i>	1, 3, 6, 7, 8, 9 (USDA) 4 (USDA), 5	2 4 (S Africa) 9 (S Africa)

not shown). The cDNA fragments 7 of the 9 serotypes have the same pattern by *BamHI* and *SacI*. These 2 enzymes respectively generate 2 and 3 cleavage fragments.

Only AHSV-9 strains cDNA are not cleaved by *SphI*. For the other 8 serotypes (AHSV-1 to -8), the patterns are similar.

AsnI generates 2 bands with the cDNA fragments 7 of the serotypes 1, 3, 6 and 8, 2 other bands with the 2 serotypes AHSV - 4 (USDA) and -9 (USDA) and 3 bands with the serotypes 5 and 2. *AsnI* gives different patterns for AHSV-7 and AHSV-4 (South Africa) strains. *HphI* does not cut the cDNA of AHSV- 2, - 4 (USDA) or - 5 strains, but it gives the same patterns for AHSV-1, -3, -4 (South Africa), -6, -8 and -9 (for both USDA and South Africa strains).

PvuII gives 2 similar DNA fragments when digesting the segments 7 of AHSV-2, -5 and -9 (USDA and South Africa) strains, but this enzyme does not cleave the cDNA segment 7 of the other serotypes.

CfoI generates the same restriction patterns for AHSV -1, -3, -6, -7, -8, -9 (USDA), for AHSV -4 (USDA), -5 strains but different profiles for the serotypes 2, 4 (South Africa) and 9 (South Africa). The restriction fragment length polymorphisms (RFLP) are identical (but different from those of the other serotypes) for AHSV-4 (USDA) and 5 strains.

CfoI gives a cleavage fragment of 550 bp with AHSV-9 (USDA) segment 7 cDNA (fig 2J, lane 8), which is not present in segment 7 AHSV-9 (South Africa) pattern (fig 2K, lane 8).

DISCUSSION

Using the 2 primers selected from the sequence data on AHSV-4 segment 7 gene, it is possible by PCR to amplify the fragment 7 cDNA, not only of AHSV serotype 4, but also of the 8 other serotypes. The identity of the amplified product was checked by studying the RFLP using 8 different restriction enzymes.

When comparing the primer sequences with all the gene sequences present in the gene "EMBL database" by computer analysis, and after testing the specificity of the primers by using other equine virus-infected cell cultures, no homology was found between the 2 primer sequences and viral genes (especially the most frequently isolated viruses from equine samples) or between the primers and cellular genes (including equine DNA extracted from cell cultures).

This method could be useful for a rapid diagnosis of the disease when suspected, and for orientating the epidemiological studies. Even when no cytopathic effect was seen in a cell culture, it was possible to amplify by PCR a genomic fragment (data not shown) and confirm the suspicion.

When comparing all the RFLP obtained from the homologous segments 7 cDNA, we were able to constitute groups of viruses according to the restriction enzymes. Table II summarizes our data and indicates viral strain groups for which segments 7 cDNA have the same pattern.

*Bam*HI and *Sac*I confirm the amplification of segment 7, but they are not useful for differentiation because all the strains have the same profiles with these 2 enzymes.

Using *Sph*I, similar segments 7 RFLP can be obtained with all AHSV serotypes except with the 2 AHSV-9 strains, of which segments 7 are not cleaved by this enzyme.

When the patterns of the fragments 7 cDNA of the different viral strains are analysed together (table I), it is possible to differentiate the group of viruses AHSV-1, 3, 6, 8 and the viruses AHSV-2, AHSV-4, AHSV-5, AHSV-7, and AHSV-9. The 4 serotypes AHSV-1, -3, -6 and -8 have the same RFLP with 6 enzymes (*Asn*I, *Hin*II, *Hph*I, *Pvu*II, *Sph*I and *Cfo*I). The strains we analysed can be grouped according to their identical patterns, but further data have to be obtained with other field isolates before a definitive conclusion regarding RFLP differentiation can be made. AHSV-2 and -5 strains have the same pattern except with the *Cfo*I enzyme: serotype 2 has a unique profile, while serotype 5 has the same profile as that of the AHSV-4 (USDA) strain. Without *Cfo*I, the 2 serotypes (-2 and -5) can be distinguished from the other serotypes and with the *Cfo*I pattern, the 2 serotypes can be differentiated from each other.

The AHSV-7 segment 7 cDNA has a unique pattern: the combination of the *Asn*I and *Hph*I patterns which are each different from those of the other viruses, allow this serotype to be identified (according to the strains studied in this paper).

AHSV-9 (USDA) strain can be differentiated from the other serotypes by using the *Sph*I restriction enzyme which does not cleave its cDNA. Both strains (the USDA and the South Africa strain) have the same profiles with *Asn*I, *Bam*HI, *Hph*I, *Pvu*II and *Sac*I. AHSV-9 (USDA and South Africa) fragments 7 cDNA have no restriction site for *Hin*II and *Sph*I, but their RFLP show one difference with the *Cfo*I enzyme pattern. This difference could be explained by the genetic variation of the South Africa strain induced by serial passages to semi-permissive non equine cell lines (as BHK 21 cells) and by serial intracerebral passages of the USDA strain in mice.

Concerning AHSV-4, the segment 7 cDNA of its South Africa vaccine strain

produced on BHK 21 cells has, for each enzyme, a pattern similar to that predicted by the fragment 7 sequence of the Spanish isolate. However, the vaccine AHSV-4 strain provided by the USDA has different restriction patterns for 4 enzymes (*AsnI*, *HinfI*, *HphI* and *CfoI*) when compared with the vaccine AHSV-4 (South Africa) strain. AHSV-4 (South Africa) has the same pattern as the expected profile of the Spanish AHSV-4 isolate determined from the sequencing data. The differences between the vaccine strain (USDA) pattern and the sequenced strain (Spanish isolate) RFLP, and the homology between the latter strain profile and the vaccine AHSV-4 (South Africa) strain pattern could be caused by the propagation of the AHSV-4 (USDA) in mice.

In shortening the delay of response in the diagnosis of an AHSV infection and in providing partial information on the involved serotype, the PCR method associated with RFLP analysis is an interesting methodology which has to be more completely studied. In addition, further information could be obtained by comparing the restriction patterns of a greater number of different isolates for each serotype, in order to evaluate more precisely the intra-serotype genetic variability. Facing the difficulty in obtaining field isolates, we are studying the consequences on the cDNA fragment 7 restriction pattern induced by numerous passages on BHK 21 and Vero cells of AHSV-4 vaccine strain produced in mice. Experiments are also in progress to apply the PCR method to the detection of the AHSV nucleic acids directly in clinical samples.

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