The Characterization of Equine Encephalosis Virus and the Development of Genomic Probes

By G. J. Viljoen and H. Huismans

Department of Biochemistry, Veterinary Research Institute, Onderstepoort 0110 and Department of Genetics, University of Pretoria, Pretoria 0002, South Africa

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SUMMARY

Equine encephalosis virus (EEV) is an orbivirus associated with a peracute illness of horses in southern Africa. The virus has now been partially purified for the first time and characterized on a molecular level. The virion is composed of 10 dsRNA segments and a protein capsid consisting of at least seven structural proteins that vary in Mr from 36000 to 120000. Partial clones of six of the dsRNA segments of EEV serotype Cascara were obtained and analysed for possible use as serotype-specific or group-specific probes in the detection of EEV dsRNA. Cloned fragments of genome segments 3, 8 and 10 were found to show high conservation of these segments, hybridizing to dsRNA from the six EEV serotypes under conditions that indicated more than 90% sequence homology. The genome segment 2-specific probe did not hybridize with dsRNA from any of the other EEV serotypes, suggesting that this segment encodes the serotype-specific antigen of EEV. Cross-hybridization of probes from genome segments 3 and 5 with dsRNA from bluetongue virus (BTV), epizootic haemorrhagic disease virus (EHDV) and African horse sickness virus (AHSV) indicated that EEV is more closely related to BTV and EHDV than to AHSV. Both probes can be used to distinguish between EEV and AHSV dsRNA.

INTRODUCTION

African horse sickness virus (AHSV) was for many years the only orbivirus of significance known to infect horses. However, in 1967 another orbivirus called equine encephalosis virus (EEV) was isolated from horses that died from an unknown peracute illness (Erasmus et al., 1970). Necropsy examinations revealed general venous congestion, fatty liver degeneration, brain oedema and highly conspicuous and sharply demarcated areas of catarrhal enteritis, especially in the distal half of the small intestine. Histopathological examination confirmed the microscopic findings (Lecatsas et al., 1973). Serological investigations revealed that widespread infections of horses with EEV had occurred during the late summer of 1967. This survey furthermore suggested that EEV had not occurred in South Africa to any appreciable extent in the preceding 10 years. Seasonal outbreaks of the disease are coupled to the presence of the suspected insect vector, Culicoides (Theodoridis et al., 1979). Outbreaks of EEV infection have furthermore been associated with equine foetus abortion during the first 5 to 6 months of gestation. An early undetected abortion could therefore be misdiagnosed as infertility in a mare.

EEV has been found to differ from the Eastern, Western and Venezuelan encephalomyelitis viruses (belonging to the alphavirus group) in that it resisted the action of lipid solvents such as chloroform (Erasmus et al., 1970). In this respect and also with regard to morphology and
cytopathology, the virus seems to belong to the orbivirus genus with a marked resemblance to viruses such as AHSV and bluetongue virus (BTV).

Six different EEV serotypes have been identified in southern Africa of which Kyalami, Bryanston and Cascara are the best known. Complement fixation tests have failed to demonstrate a relatedness between any of the EEV serotypes and other orbiviruses such as BTV, AHSV or epizootic haemorrhagic disease virus (EHDV) (Erasmus et al., 1978).

Electron microscopic studies on thin sections of EEV-infected baby hamster kidney cells revealed that mature virus particles are released from the granular inclusion bodies seen in the cytoplasm. The cytopathology is further characterized by a swelling of the rough endoplasmic reticulum and by the presence of inclusion bodies in the mitochondrial matrix (Lecatsas et al., 1973).

In order to distinguish rapidly and efficiently between horses infected with AHSV and EEV, it is necessary to differentiate between these viruses not only on a serological level but also on a molecular and genomic level, and the availability of group-specific genomic probes could be very useful in this respect. Such probes would also greatly enhance the study of the pathology and epidemiology of EEV infections. No molecular characterization of EEV has as yet been carried out. It is not known to what extent EEV resembles AHSV or which of the genome segments would be suitable as a group-specific or serotype-specific probe. This paper reports the characterization of the dsRNA and protein components of the virion, and the analysis of six different EEV genome-specific clones as probes for the detection of EEV dsRNA.

**METHODS**

**Cells.** Three cell lines were used for this investigation: BHK-21, C6/36 (*Aedes albopictus*) and Vero cells. Origins of these cell lines and media and methods for the preparation of monolayer cell cultures have previously been described (Verwoerd et al., 1967; Oellerman, 1970; King & Alders, 1985). Although the yields of EEV Cascara in C6/36 cells were approximately 10% higher than in BHK-21 cells, the latter cells were the preferred cell line due to the ease of handling and were grown either in Roux flasks or in glass roller flasks.

**Virus.** The origins of the BTV, EHDV and AHSV serotypes used in this investigation have been described (Huismans & Cloete, 1987). The EEV serotypes Cascara, Kaaplaas, Langeberg, Kyalami and Gamil were isolated from equine spleen, Bryanston from equine lung. All viruses were plaque-purified and stored in a freeze-dried form at −20°C. The viruses were routinely grown in BHK-21 cells using an inoculum which was never more than five serial passages removed from the plaque-purified virus isolate. In the unpurified form the virus is stable for up to 12 weeks at 4°C without excessive loss of infectivity. Plaque assays were performed in monolayers of Vero cells grown in 60 mm Petrie dishes (Oellerman, 1970). Large clear plaques were obtained.

Virus was partially purified by a Triton X-100 purification method (Huismans et al., 1987b) and stored at 4°C at a concentration of 2.5 to 3 mg/ml. EEV dsRNA was extracted from partially purified virus as described by Verwoerd et al. (1970).

**Electrophoresis.** Protein electrophoresis was carried out as described by Laemmli (1970). Viral proteins were separated on 15% polyacrylamide slab gels for 16 h at 200 V. Gels were stained in a 0.2% Serva blue staining solution. dsRNA was separated on 4% polyacrylamide gels at 100 mA, and 70 V for 16 h using a Tris–acetate–EDTA–SDS buffer (Loening, 1967). The gels were stained with ethidium bromide (1 l.tg/ml) and the dsRNA bands were visualized on a u.v. transilluminator.

**Cloning of dsRNA.** Cloning of EEV Cascara dsRNA was carried out by a modification of the method of Cashdollar et al. (1984). Preparations of between 40 and 50 μg purified EEV Cascara dsRNA were denatured with 10 mm-methylmercuric hydroxide and 3' poly(A) tracts added (Sippel, 1973). cDNA was synthesized and fractionated on alkaline sucrose gradients (Huismans & Cloete, 1987). The pooled cDNA fractions were characterized on alkaline agarose gels (McDonell et al., 1977), dC-tailed and cloned into dG-tailed *PstI*-cut pBR322 as described by Huismans et al. (1987a).

**Characterization of recombinant plasmids.** Plasmids were purified by an alkaline lysis method (Birnboim & Doly, 1979). Genome assignments were carried out by Northern blot analysis as described by Huismans & Cloete (1987). Probes were labelled by nick translation (Rigby et al., 1977; Maniatis et al., 1982) using a nick translation kit (Bethesda Research Laboratories).

**Hybridization.** Prehybridization and hybridization of the blots were carried out as described by Huismans et al. (1987a). Dot spots of dsRNA were prepared by immobilizing 100 ng amounts of purified, MMOH-denatured, dsRNA on Hybond-N nylon membranes (Amersham) using a 96-well dot-spot apparatus from Bio-Rad. Hybridization stringency was regulated as indicated in the text by changing the conditions under which the blots were washed.
**RESULTS**

**Characterization of EEV proteins and dsRNA**

EEV was purified using the Triton X-100 method described by Huismans *et al.* (1987b) and yields of up to 0.5 mg virus/10^8 cells were obtained. The EEV structural proteins were characterized by comparing BTV and AHSV proteins on SDS-PAGE. The result is shown in Fig. 1. The Mr of the structural proteins varied between 36000 and 120000. The protein fractionation pattern of EEV on PAGE gels closely resembled that of the other two orbiviruses. The four major capsid proteins VP2, VP3, VP5 and VP7 were easily identified as were the minor proteins VP1 and VP4. VP2 and VP5 are presumed to be the proteins in the outer capsid as they were lost or reduced in relative amount after centrifugation on CsCl density gradients (results not shown). The minor band that migrated immediately above VP7 is likely to represent VP6 of EEV. However, another minor polypeptide that migrated in a position between VP6 and VP7 (X in Fig. 1) was observed several times and could also represent VP6 or a non-structural or cellular protein that copurifies with EEV.

Electrophoretic separation of the dsRNA genome of AHSV strain 3, BTV strain 4 and EEV Cascara on PAGE gels is shown in Fig. 2(a). The EEV genome is composed of 10 genome segments and the PAGE separation profile of the segments differed considerably from those of BTV and AHSV. PAGE gels are, however, not suitable for estimating an accurate size for dsRNA segments (Pedley *et al.*, 1988) so the different dsRNA isolates were also analysed on agarose gels. The separation of dsRNA segments from the six EEV serotypes on a 1% agarose gel is shown in Fig. 2(b). dsRNA from BTV-4, AHSV-3 and EHDV New Jersey were included as controls. There were differences in the dsRNA profiles from BTV, AHSV, EHDV and EEV. The EEV dsRNA profile most closely resembled that of AHSV. With the exception of segment 6, most of the cognate AHSV and EEV genome segments were of about the same size. The dsRNA profiles of the six EEV serotypes on agarose gels were indistinguishable. The approximate size of the different EEV genome segments was calculated from agarose gels using the known sizes of the BTV segments as a reference and the results are shown in Table 1.

![Fig. 1. Comparison of the gel electrophoretic fractionation of the capsid polypeptides of sucrose gradient-purified EEV Cascara (lane 1) and AHSV-3 (lane 2). The structural proteins VP1 to VP7 are as indicated. Also shown are the polypeptides of a semi-purified preparation of BTV (a particulate fraction) that contains the two major non-structural proteins NS1 and NS2 (lane 3). Electrophoresis was carried out on a 15% SDS-PAGE gel for 16 h at 200 V.](image-url)
Fig. 2. (a) Separation by PAGE of 5 μg quantities of the dsRNA genome segments of AHSV-3 (lane 1), EEV Cascara (lane 2) and BTV-4 (lane 3). Electrophoresis was carried out for 16 h at 70 V on a 4% gel. (b) 1% Agarose gel electrophoresis of 1 μg amounts of the various dsRNAs: lane 1, EEV Cascara; lane 2, BTV-4; lane 3, EHDV New Jersey; lane 4, AHSV V-3; lane 5, EEV Gamil; lane 6, EEV Kaalplaas; lane 7, EEV Langeberg; lane 8, EEV Bryanston; lane 9, EEV Kyalami. EEV genome segments were numbered 1 to 10 in order of decreasing size.

Table 1. A comparison of the size of EEV dsRNA segments and the genome-specific cloned inserts

<table>
<thead>
<tr>
<th>Genome segment</th>
<th>Size* (bp)</th>
<th>Size of cloned segments† (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3900</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>3220</td>
<td>2900</td>
</tr>
<tr>
<td>3</td>
<td>2750</td>
<td>600</td>
</tr>
<tr>
<td>4</td>
<td>2020</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1750</td>
<td>300</td>
</tr>
<tr>
<td>6</td>
<td>1570</td>
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</tr>
<tr>
<td>7</td>
<td>1080</td>
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<td>8</td>
<td>1080</td>
<td>600</td>
</tr>
<tr>
<td>9</td>
<td>1080</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>710</td>
<td>800</td>
</tr>
</tbody>
</table>

* Calculated from the results shown in Fig. 2(b), using the electrophoretic migration of the known BTV dsRNA segments as a reference.
† Calculated from the electrophoretic mobility on 1% agarose of the PstI-excised inserts. Linear DNA electrophoretic markers were used as a control.

Cloning of EEV dsRNA segments

Genomic probes of the different EEV dsRNA segments were obtained by shot-gun cloning of EEV Cascara dsRNA. Cloning was carried out as described in Methods. Recombinant plasmids were characterized by electrophoresis and a selection of 32P-labelled plasmids were hybridized to a blot of electrophoretically separated EEV Cascara dsRNA (Fig. 3). Cloned
fragments of genome segments 2, 3, 5, 6, 8 and 10 were obtained. Their sizes were estimated and the results in Table 1 show the variation in size from approximately 300 bp for genome segment 5 to 2000 bp for genome segment 2. The cloned genome fragment 10 was approximately 800 bp and therefore probably represented a full-length clone.

**Dot blot hybridization of EEV Cascara-specific DNA clones**

In order to identify a suitable EEV group-specific probe it was first of all important to identify those genome segments which are highly conserved in the EEV serogroup. This was carried out by hybridizing each of the EEV Cascara genomic clones to dot spots of denatured dsRNA from each of the six different EEV serotypes under conditions of either high or low hybridization stringency. The high stringency conditions were selected to detect a homology of more than 90% whereas the low stringency identified hybrids with a nucleic acid homology of more than 75%. The results are shown in Fig. 4.

Three of the six genome segment probes (3, 8 and 10) were highly conserved and hybridized with all EEV serotypes under high stringency conditions. Other genome segments such as 5 and 6 are less highly conserved amongst the members of the EEV serogroup. The genome segment that showed the largest variation was segment 2 which did not hybridize with any of the heterologous serotypes at a high stringency. When the stringency was lowered to detect
Fig. 4. Autoradiograph of the hybridization of six $^{32}$P-labelled EEV Cascara serotype-specific DNA probes to 100 ng quantities of denatured dsRNA from the six EEV serotypes immobilized on a Hybond-N membrane. The membranes were prepared and hybridized as described in Methods. The blots were washed with high stringency (0.1 x SSC) (a) and low stringency (1 x SSC) (b) buffers at 65 °C. The letters indicate the EEV serotypes: (a) Cascara, (b) Gamil, (c) Kaalplaas, (d) Langeberg, (e) Bryanston, (f) Kyalami. The numbers on the left indicate the different genome segment probes.

Fig. 5. Autoradiograph of the hybridization of the $^{32}$P-labelled genome segment 5 (a) and 3 (b) probes of EEV Cascara with 100 ng amounts of dsRNA from (a) EEV Cascara, (b) BTV-4, (c) AHSV-3 and (d) EHDV New Jersey. Hybridization was carried out as described in Methods and the stringency of hybridization was varied by using SSC concentrations that varied from 0.1 x SSC to 5 x SSC and 0.01% SDS, during the washing, that is (1) 5 x SSC, (2) 3 x SSC, (3) 1 x SSC, (4) 0.5 x SSC and (5) 0.1 x SSC.

Homology of more than 75%, hybridization of the EEV Cascara segment 2 probe to EEV Kaalplaas dsRNA was observed. The homology to cognate genes of the other serotypes is presumed to be less than 75%.

A second important prerequisite of an EEV-specific probe is that it should be able to distinguish between EEV and other orbiviruses. The two EEV probes that were investigated in this respect were those of segments 3 and 5, thought to be related to BTV genes 3 and 5 which in turn have previously been recommended for use as BTV serogroup-specific probes (Roy et al., 1985; Huismans & Cloete, 1987). In the experiment shown in Fig. 5, $^{32}$P-labelled probes of segments 3 and 5 of EEV Cascara were hybridized to dot spots of dsRNA from EEV Cascara AHSV-3, BTV-4 and EHDV New Jersey. The hybridization stringencies were varied to detect nucleic acid homologies in the range of 60% to >90%.
There was no hybridization of the EEV genome segment probes with dsRNA from any of the other orbiviruses under conditions that required a homology of more than 80% (0.5 × SSC, 65 °C). At a lower stringency, cross-hybridization with BTV and EHDV dsRNA was observed, suggesting that the nucleic acid homology of the cognate segment 3 genome segments was approximately 75 to 80%. Hybridization with AHSV dsRNA was observed only after a further reduction in stringency. The segment 5-specific EEV probe hybridized with AHSV dsRNA only under very low hybridization stringency conditions. The homology between AHSV and EEV segment 5 genes is therefore probably no more than about 65%.

**DISCUSSION**

Equine encephalosis virus closely resembles AHSV with respect to morphology, cytopathology and the manifestation of clinical symptoms in horses (Erasmus et al., 1970). This often creates difficulties in distinguishing between the two viruses for diagnostic purposes, a problem that motivated an investigation of the molecular composition of EEV in comparison to that of AHSV and the development of nucleic acid probes that could distinguish between the two viruses.

As a first step in the investigation, EEV was purified using standard procedures developed for BTV (Huismans et al., 1987b) and yields of approximately 0.5 mg virus/10⁶ cells were obtained. However, after the sucrose gradient centrifugation step the virus appeared to be still associated with a few minor proteins of unknown origin. Some are perhaps small amounts of non-structural proteins as has been found for BTV (Mertens et al., 1987; Eaton et al., 1988). BTV non-structural proteins NS2 and NS1 have both been found to be associated with BTV purified on sucrose gradients. Further purification of EEV on CsCl density gradients (pH 8.0) was not successful; the virus was found to be unstable at the high salt concentration as seen by the loss of some of the major capsid polypeptides. The protein profile of EEV resembles that of a typical orbivirus with four major and at least three minor protein components which range in size from 36000 to 120000. The separation profile is different from that of AHSV and BTV but not sufficiently different to be used as a means of identifying EEV.

A comparison of EEV and AHSV dsRNA on PAGE gels also indicated characteristic differences. However, as shown by Pedley et al. (1988) for BTV, the separation of dsRNA genome segments by PAGE is not a good reflection of the size of the respective RNA segments. A much better size estimate is obtained on agarose gels. It has been reported that all serotypes of BTV (Pedley et al., 1988), EHDV (Brown et al., 1988) and other orbiviruses (Bodkin & Knudson, 1985; Gonzalez & Knudson, 1987, 1988) have similar dsRNA profiles on agarose gels. Similar results were obtained with the six EEV serotypes. The EEV dsRNA profile on agarose gels differed from that of EHDV and BTV but the EEV and AHSV dsRNA profiles were very similar, underlining the necessity to distinguish between the two viruses by another method. One such approach is the use of EEV-specific nucleic acid probes. Such probes can also be used to study the variation amongst cognate genes in the EEV serogroup and to investigate the relatedness between EEV and other orbiviruses such as AHSV.

Cloned fragments of genome segments 2, 3, 5, 6, 8 and 10 of EEV Cascara were analysed by hybridization to dot spots of dsRNA from the six different EEV serotypes. The results indicated that EEV genome segments 3, 8 and 10 are highly conserved. The homology of cognate genes amongst the six EEV serotypes appeared to be more than 90%. The segment 3 probe of EEV Cascara was also hybridized to BTV, EHDV and AHSV dsRNA. The results indicated a homology of approximately 75 to 80% between segment 3 of EEV and the cognate genes of EHDV and BTV respectively. Cognate segment 3 genes of EEV and AHSV showed less homology (approximately 65 to 75%), whereas the homology between cognate genome segments 5 of these two viruses was even lower, supporting the suggestion that EEV is more closely related to BTV and EHDV than to AHSV. In the interpretation of the results it should be kept in mind that most of the probes were not full-length DNA copies of corresponding genome segments. The results obtained with the very short probes are therefore not necessarily always a true reflection of that of the full-length genome segment.
Both segment 3 and 5 probes can both be used to distinguish between EEV and AHSV under conditions of moderate hybridization stringency. For in situ hybridization the use of the segment 5 probe is recommended because segment 5 mRNA is expressed in very much higher amounts than segment 3 mRNA and this increase in the amount of target RNA makes the segment 5 probe much more sensitive (G. J. Viljoen, unpublished results). On the other hand segment 5 is not as highly conserved as segment 3 in the EEV serogroup which limits its use as a serogroup-specific probe under conditions of high hybridization stringency.

The genome 2-specific probe of EEV Cascara did not hybridize with dsRNA from heterologous EEV serotypes under conditions of high stringency, and at a lower stringency which requires a homology of more than 75%, it hybridized only with EEV Kaalplaas. B. J. Erasmus (personal communication) has found that there is a significant level of cross-neutralization between EEV Cascara and EEV Kaalplaas. These observations suggest that segment 2 of EEV encodes the protein involved in the induction of serotype-specific neutralizing antibodies. Hybridization with a larger variety of EEV Cascara isolates should provide further evidence.

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Equine encephalosis virus genomic probes


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