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Recovery of Swedish *Equine arteritis viruses* from semen by cell culture isolation and RNA transfection

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Abstract

Recovery of infectious *Equine arteritis virus* (EAV) from the semen of persistently infected Swedish stallions was attempted by classical cell culture isolation and by transfection of extracted total RNA. Whereas virus from semen samples stored for several months at −20 °C or from extended semen could only be recovered by transfection of extracted RNA, isolation in cell culture was achieved readily with fresh, unextended semen stored at −70 °C or directly used after sampling. In parallel, the viruses were examined in the variable region of the large glycoprotein GP5 by nested RT-PCR and direct nucleotide sequencing. The resulting sequences were placed into a large phylogenetic tree from this region, demonstrating that Swedish strains belonged to very diverse phylogenetic groups. This represents the first report of recovery of infectious EAV from archived semen samples by RNA transfection.

Keywords: Equine arteritis virus; Isolation; Recovery; RNA transfection

1. Introduction

Equine arteritis virus (EAV) represents the prototype member of the *Arteriviridae* family within the order *Nidovirales* (Cavanagh, 1997), that also contains porcine reproductive and respiratory syndrome virus (PRRSV), simian haemorrhagic fever virus (SHFV), and lactate dehydrogenase-elevating virus of mice (LDV).

The genome is a 3′-polyadenylated, positive-stranded RNA of approximately 12.7 kb (den Boon et al., 1991), from which seven open reading frames (ORFs 2a, 2b, and 3–7) are transcribed in a nested set of mRNAs (de Vries et al., 1997), and where the 5′ three-quarters are occupied by ORFs 1a and 1b. The gene for the large glycoprotein GP5 (ORF5) is highly variable in sequence (Stadejek et al., 1999), making it highly suitable for phylogenetic studies.

Exposure to EAV usually results in a mild or subclinical infection in immunocompetent adult animals but can also cause abortion in pregnant mares and death in newborn foals (reviewed in Timoney and McCollum, 1993). A considerable number of stallions become long-term carriers of the virus, playing a major epidemiological role in the dissemination and perpetuation of EAV between outbreaks (Timoney et al., 1986, 1992; Klingeborn et al., 1992) since they shed EAV in their semen and transmit the infection sexually to a high percentage of susceptible mares (Timoney et al., 1987; Hyllseth et al., 1991; Glaser et al., 1996).

EAV is widespread in most countries with dense horse populations, including Sweden (Metcalf, 2001). The disease often remains undetected or the cause of the disease is not identified correctly, respectively, because strains of EAV circulating today are considered as low or even non-virulent. No abortions could be attributed to EAV in Sweden so far, but outbreaks of EAV do occur in other countries than Sweden (Szeredi et al., 2003; Larsen et al., 2001; Del Piero et al., 1997; Eichhorn et al., 1995; Monreal et al., 1995; Wood et al., 1995; Vaala et al., 1992), and

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there is no possibility of predicting in which direction within the possible spectrum of virulence the virus will evolve. Therefore it is of critical importance to gain more knowledge about the strains of EAV circulating today and to analyse and compare their in vitro and in vivo properties.

Isolation of EAV in cell culture can be achieved readily from freshly collected semen samples or when they have been stored properly. However, recovery from extended semen, collected for artificial insemination, or from semen stored at −20 °C or less, is very difficult if not impossible by classical cell culture inoculation. However, genetic analysis of the whole genome, or studies of growth characteristics in vitro are facilitated by large quantities of infectious virus. Therefore, the recovery of infectious EAV was attempted both by the conventional cell culture technique and also by transfection of extracted RNA into susceptible cell lines of all Swedish samples available. This report also describes the phylogenetic classification of these viruses by nucleotide sequencing of the variable part of the ORF5.

2. Materials and methods

2.1. Semen samples

Fresh semen samples were collected from Swedish stallions (sample S6 in Table 1), sent immediately to the laboratory on wet ice, and either stored at −70 °C until use or centrifuged directly to separate the sol phase from the sperm rich fraction. Extended semen samples were either retrieved from −20 °C freezers, or obtained at ambient temperature in the course of a routine surveillance programme (samples S2, S4, and S7 in Table 1).

2.2. Cells and control virus

Rabbit kidney (RK13) and baby hamster kidney (BHK-21) cells were maintained in EMEM with 10% FCS. Equine embryonic lung cells (EEL) were grown in EMEM + Tricin with 10% FCS. All cells were grown in a humidified atmosphere containing 5% CO_2 at 37 °C.

2.3. Virus isolation in cell cultures

Semen samples were sonicated at 14μ for three times 30 s in 1-min intervals on ice. After centrifugation at 1000 × *g* for 10 min at 4 °C, the sol fraction was transferred to a new tube, stored at −70 °C or directly used.

Confluent monolayers of BHK-21, EEL or RK13 cells in six-well plates were inoculated with undiluted or diluted semen.

Table 1

Isolate name	Year	Sample	Breed
S1 (AF099842)	1989	Semen	Swedish warmblood
S2	1999	Semen	Trotter
S4	1999	Semen	Trotter
S6	2000	Semen	Trotter
S7	2000	Semen	Trotter

After incubation at 37 °C for 1 h the inoculum was removed and replaced with growing medium containing 0.75% (w/v) CMC (carboxyl-methyl cellulose). Cells inoculated with undiluted semen were monitored at 15 min intervals and the inoculum was replaced as soon as rounding of the cells was observed.

Cultures were monitored daily for the appearance of a cytopathic effect (CPE). Negative cultures were freeze/thawed twice and passaged onto fresh cells in six-well plates until the appearance of a CPE or for a maximum of three passages.

2.4. RNA extraction

Total RNA was extracted from 250 μl semen with TRIzol (Gibco/BRL) according to the manufacturer's instructions. The resulting pellet was dissolved in 50 μl of DEPC-treated water; 5 μl thereof were used in the reverse transcription (RT) reaction.

2.5. RNA transfection

One or ten micrograms of extracted RNA was transfected into cells by electroporation with a BioRad Gene Pulser I apparatus. Four million BHK-21, EEL or RK13 cells were trypsinized, washed twice with PBS and resuspended in 400 μl cold PBS. After mixing with RNA the cells were transferred to cold 0.4 cm cuvettes and subjected immediately to one pulse at 300 V and 500 μF. Following electroporation the cells were rested for 5 min at room temperature, resuspended in EMEM with 10% FCS and seeded into the wells of a six-well plate at an appropriate concentration.

2.6. Reverse transcription and PCR

All semen and cell culture samples were first tested by nested RT-PCR in the nucleocapsid-coding region (Belák et al., 1994).

Reverse transcription was carried out as described previously (Stadejek et al., 1999). The nested PCR for phylogenetic analysis was performed on PCR-positive samples with the slightly modified first round primers GL105F (nucleotide positions 11250-11271 of the full-length sequence of the Bucyrus strain, GenBank accession number NC_002532) and GL673R (11840-11818), and the second round primers CR2 (11272-11294) and EAV32 (11836-11814), described previously (Stadejek et al., 1999), flanking a 546 and 519 nucleotide fragment, respectively, of the GP5 protein.

Amplification was slightly modified from the previous description of this PCR procedure (Stadejek et al., 1999): the reaction mixture comprised 5 μl of 10 × PCR buffer II (Perkin-Elmer); 1.5 mM $MgCl_2$; 200 μM dNTP (each); 20 pmol of GL105 and of GL673 primers; 1 U Taq DNA polymerase and 5 μl of cDNA. Amplification involved 30 cycles of denaturation at 94 °C for 45 s, primer annealing at 55 °C for 1 min and primer extension at 72 °C for 1.5 min.

The nested amplification cycle was undertaken using a 50 μl reaction volume. The reaction mixture comprised 5 μl of 10 × PCR buffer II (Perkin-Elmer); 1.5 mM $MgCl_2$; 200 μM dNTP (each); 20 pmol of CR2 and of EAV32 primers; 1 U Taq DNA polymerase and 5 μl of the first PCR product. Amplifica-