JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 1994, p. 658–665
0095-1137/94/$04.00+0

Vol. 32, No. 3

# Detection of Equine Arteritis Virus following Amplification of Structural and Nonstructural Viral Genes by Reverse Transcription-PCR

G. ST-LAURENT, G. MORIN, AND D. ARCHAMBAULT*

*Université du Québec à Montréal, Département des Sciences Biologiques, P.O. Box 8888, Station A, Montréal, Québec, Canada H3C 3P8*

Received 9 September 1993/Returned for modification 21 October 1993/Accepted 10 December 1993

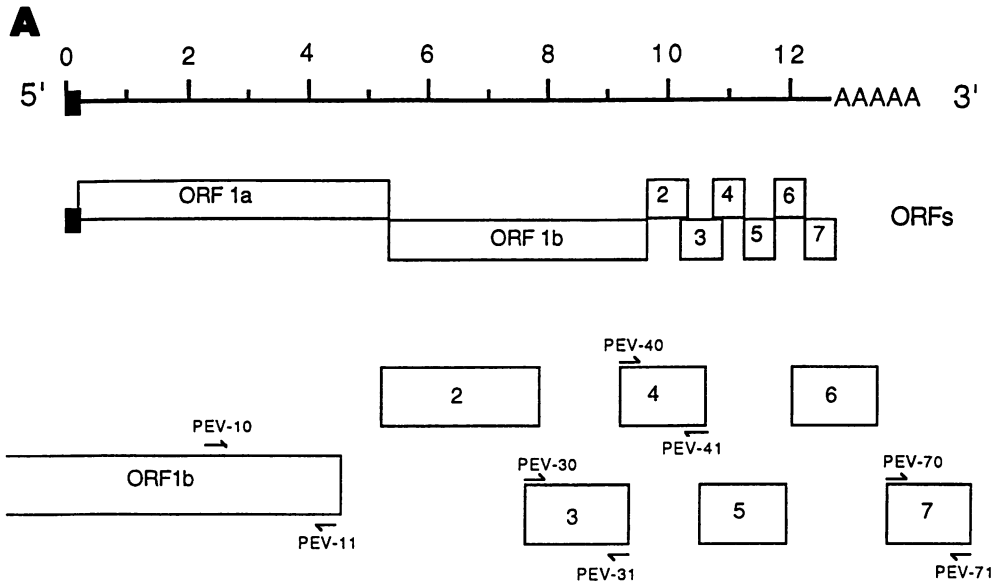
**A reverse transcription (RT)-PCR assay was developed for the detection of equine arteritis virus (EAV) in cell culture supernatant and in horse semen. Four different sets of oligonucleotide primers complementary to sequences located in the 3′ end of the polymerase gene (open reading frame [ORF] 1b) and to sequences representing the entire ORFs 3, 4, and 7, which encode for nonstructural (ORFs 3 and 4) or viral nucleocapsid (ORF 7) proteins, were compared for their abilities to amplify the targeted EAV sequences by the RT-PCR procedure. The sensitivities of the RT-PCR for amplification of EAV sequences located in the 3′ end of ORF 1b and ORF 4 were 2 median tissue culture infective doses ($TCID_{50}$s) of viral particles in the EAV-infected cell culture supernatant for both ORFs and 20 and 200 $TCID_{50}$s of viral particles, respectively, in virus-containing horse semen. The sensitivities were much lower when primers complementary to ORFs 3 and 7 were used in the RT-PCR, with a minimum detection limit of only $2 \times 10^4$ $TCID_{50}$s of viral particles in virally infected cell culture supernatant, as determined by analyzing the resulting RT-PCR products on ethidium bromide-stained agarose gels. The specificities of the RT-PCR assays for all primer sets tested were confirmed when the amplified cDNA products of the expected size reacted positively with the corresponding virus-specific digoxigenin-labeled cDNA probes in the chemiluminescence assays. Although the sensitivity of the RT-PCR for amplification of ORF 3 and 7 sequences was lower, all sets of primers were capable of amplifying several cell culture-adapted EAV field isolates when the virus was present in high enough quantities in the test sample. When horse semen samples were analyzed for the presence of EAV by the RT-PCR with primers specific to the ORF 1b 3′ end and ORF 4 sequences and by virus isolation in cell cultures, there was 100% concordance among the assays. The RT-PCR assay targeting the 3′ end of ORF 1b and/or ORF 4 of EAV RNA may be an alternative to conventional methods for the diagnosis of EAV infection in horses.**

Equine arteritis virus (EAV), the etiologic agent of equine viral arteritis, was initially isolated in Bucyrus, Ohio, from a fetus aborted during an endemic infection in standardbred horses (10). The virus, which is restricted to the *Equidae*, is widespread in the horse population (19). The symptomatology of EAV infection varies widely from a subclinically silent form to overt clinical signs which generally include pyrexia, conjunctivitis, lacrimation, nasal discharge, and edema (18). In infected pregnant mares, abortion is common. The respiratory route appears to be a major means of EAV transmission to susceptible horses in contact with acutely infected horses. However, a carrier state exists in seropositive stallions in which EAV is persistently shed in semen (26). The infected stallions thus infect mares at the time of breeding; the mares, in turn, are capable of transmitting the virus via the respiratory route to susceptible horses with which they come into contact.

EAV is a nonarthropod-borne virus that has been classified as a member of the genus *Arterivirus* within the family *Togaviridae* (27). The EAV particle varies from 50 to 70 nm in diameter and consists of an icosahedral core of 35 nm surrounded by an envelope which carries ring-like structures with a diameter of 12 to 15 nm. The viral genome is a positive, polyadenylated, single-stranded RNA of 12.7 kb (7). During EAV replication, a 3′-end coterminal nested set of seven virus-specific RNAs (open reading frames [ORFs] 1 to 7) is produced (Fig. 1A); these range in size from genomic length to 0.8 kb. Each ORF is preceded by the sequence motif 5′-UCAAC-3′, which is involved in the formation of six subgenomic EAV RNAs with a common leader sequence of 207 nucleotides derived from the extreme 5′ end of the viral genome (8). On the basis of the data obtained from viral gene expression experiments, the virion contains four structural proteins: a nucleocapsid of 14 kDa encoded by ORF 7 and three envelope proteins of 16, 25, and 30 to 42 kDa encoded by ORF 6, ORF 2, and ORF 5, respectively (7, 9). ORF 2- and 5-encoded envelope proteins are glycosylated. EAV ORF 1, which consists of two overlapping ORFs (ORFs 1a and 1b), encodes for the viral replicase. ORFs 3 and 4 direct the synthesis of a glycoprotein of 45 kDa and a labile protein of 16 kDa, respectively, as demonstrated by in vitro expression experiments (9). The ORF 3- and 4-encoded products are believed to be EAV nonstructural proteins.

The diagnosis of EAV infection is based on virus isolation in cell cultures or serum antibody determination of virus-exposed animals by complement fixation or virus neutralization tests (3, 12, 13, 24). Because EAV-infected asymptomatic stallions shed the virus in their semen and, thus, act as a reservoir for future disease outbreaks, semen samples are screened for EAV by sensitive but time-consuming virus isolation attempts in cell cultures. PCR is a rapid technique by which the nucleic acid in any sample can be specifically amplified by up to $10^6$-fold prior to attempting to detect it (22). Moreover, over the past few years, a reverse transcription (RT) step has been used in conjunction with the PCR amplification, in the so-called RT-PCR, for the detection of the viral RNA genome in

* Corresponding author.




B

| Primer symbol | Sense | Sequence 5' to 3' | Genome location bases | Product size bp |
|---|---|---|---|---|
| PEV-10 | + | GAGGATCCCACTTCATCT | 9130-9147 | 535 |
| PEV-11 | - | AATGGTCTGCACTGAGGT | 9665-9648 | |
| PEV-30 | + | CTCTAGAATGGGTCGTGCCTACAG | 10289-10305 | 494 |
| PEV-31 | - | CAAGCTTAAATTACGAGCCTCTGCA | 10783-10766 | |
| PEV-40 | + | CTCTAGAATGAAGATCTACGGCTGC | 10683-10700 | 482 |
| PEV-41 | - | CAAGCTTCCCAAAGCAGAATAGCA | 11165-11148 | |
| PEV-70 | + | CTCTAGAATGGCGTCAAGACGATC | 12296-12312 | 342 |
| PEV-71 | - | CAAGCTTTATCCACGTCTTACGGC | 12638-12622 | |

FIG. 1. (A) Schematic representations of the genome of EAV and the oligonucleotide primers used in the RT-PCR assays. The solid box refers to the leader sequence, and the open boxes represent ORFs 1 to 7. ORFs 1a and 1b encode the viral polymerase gene. EAV structural proteins are encoded by ORFs 2, 5, and 6 (membrane proteins) and ORF 7 (nucleocapsid protein). ORFs 3 and 4 are believed to encode viral nonstructural proteins. The arrows indicate the locations of the primers. (B) Sequences of the oligonucleotide primers used in RT-PCR. The underlined nucleotides are short 5′ extensions which contain the restriction enzyme sites *Hin*dIII (AAGCTT) or *Xba*I (TCTAGA) that were incorporated for subsequent cloning of the genes (ORFs 3, 4, and 7) in an expression vector. The melting temperatures of all oligonucleotide primers, including only sequences (nonunderlined) derived from the EAV genome, varied from 52 to 55°C.

biological samples. In our study we took advantage of that technique and tested and compared the abilities of different sets of EAV genome-specific oligonucleotide primers to be used in the RT-PCR for virus detection, targeting the nucleotide sequences of ORFs 3, 4, and 7 and the 3′ end of ORF 1b of the viral genome.

## MATERIALS AND METHODS

**Virus and cell culture.** The Bucyrus strain of EAV (10) propagated in rabbit kidney (RK-13) cells was used to establish the RT-PCR assays. The cells were grown at 37°C in an atmosphere of 5% $CO_2$ supplemented with 10% fetal bovine serum in minimum essential Eagle's medium (MEM; Bethesda Research Life Technologies, Gaithersburg, Md.). Confluent 2-day-old cell cultures were inoculated with the virus at a multiplicity of infection of 0.01 and were further incubated with 2% fetal bovine serum in MEM until 80 to 100% of the cells exhibited a cytopathic effect. After one cycle of cell freezing and thawing, the cell culture supernatant was centrifuged at 5,000 × *g* for clarification. Virus was then pelleted by centrifugation (100,000 × *g* for 3 h) at 5°C over a 25% (wt/vol) sucrose cushion. The virus pellet was resuspended in TNE buffer (50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA [pH 7.4]). The viral titers were determined and calculated as the median tissue culture infective dose ($TCID_{50}$) per milliliter (1).

**RNA extraction.** The virion RNA was extracted by the guanidium isothiocyanate method (6). Briefly, 500 μl of 4 M guanidium isothiocyanate, 50 μl of 2 M sodium acetate (pH 4.0), 500 μl of water-saturated phenol, and 100 μl of chloroform were added to 100 μl of the sample, with thorough mixing after the addition of each reagent, and the mixture was left on

ice for 15 min. Thereafter, the mixture was microcentrifuged for 20 min at 5°C, and the upper aqueous phase was transferred to a fresh tube. Viral RNA was precipitated with 500 μl of cold isopropanol for 1 h at −20°C. After centrifugation, the pellet was resuspended in 150 μl of 4 M guanidium isothiocyanate. The suspension was then precipitated with isopropanol, and the resulting RNA pellet was washed with 75% ethanol, dried, and resuspended in 20 μl of diethylpyrocarbonate-treated water. The RNA samples were kept at −70°C until use.

**Oligonucleotide primers, cDNA synthesis, and PCR.** The oligonucleotide primers shown in Fig. 1A and B and designed according to the published sequence of the EAV Bucyrus strain genome (7) were synthesized with a Gene Assembler Plus DNA Synthesizer (Pharmacia, Uppsala, Sweden). The melting temperatures of the oligonucleotide primers varied from 52 to 55°C.

For cDNA synthesis, the reaction mixture was incubated at 70°C for 10 min and was chilled on ice prior to the addition of the reverse transcriptase enzyme and RNasin. RT of EAV RNA was carried out at 37°C for 15 min and at 42°C for 90 min in 1× *Taq* buffer (10× *Taq* buffer is 500 mM KCl, 100 mM Tris-HCl [pH 8.3], 1% Triton X-100) containing 0.5 mM (each) deoxynucleoside triphosphates and to which 3 μl of A buffer (400 mM Tris-HCl [pH 8.3], 50 mM $MgCl_2$), 8 U of avian myeloblastosis virus reverse transcriptase (Promega Corporation, Madison, Wis.), 17 U of RNasin (Pharmacia), 4 μl of RNA sample, and 20 pmol of antisense primer (Fig. 1B) were added to a final reaction volume of 30 μl. Thereafter, the mixture was incubated at 95°C for 5 min to inactivate the avian myeloblastosis virus reverse transcriptase, and the following reagents were added: 50 pmol of each sense and antisense primer, 0.2 mM (each) deoxynucleoside triphosphates, 2.5 U of *Taq* polymerase (Bio/Can Scientific, Inc., Mississauga, Ontario, Canada), and 10 μl of 10× *Taq* buffer to a final reaction volume of 100 μl. The cDNA was then amplified by 30 successive cycles of denaturation at 95°C for 1 min, primer annealing at 50°C for 1 min, and DNA chain extension at 72°C for 2 min with a programmable thermal cycler (Temp.Tronic; Barnstead/Thermolyne, Dubuque, Iowa). All reagents used in the RT-PCR procedure including the buffer and the primer solutions were routinely tested before use for possible EAV contamination by running mock RT-PCRs. The conditions used in the RT-PCR procedure described above were obtained from preliminary experiments in which RT-PCR assays with each set of primers were optimized by varying the annealing temperatures and the primer and $MgCl_2$ concentrations in the reaction mixtures (data not shown). The denaturation and extension times and temperatures described above for all primer pairs were also found to be suitable for the cDNA amplification step.

**Preparation of EAV cDNA probes.** The amplified cDNA fragments from the 3′ end of ORF 1b (535 bp), ORF 3 (494 bp), ORF 4 (482 bp), and ORF 7 (342 bp) (Fig. 1B) of the EAV genome were cloned into the PCR II TA cloning vector for probe preparation by the method described by the supplier (Invitrogen, San Diego, Calif.). Recombinant plasmids were then isolated from cultured bacteria by the alkaline lysis method (16). The cloned cDNAs were sequenced by the chain termination sequencing method and were analyzed for EAV sequence confirmation (7). The cDNA fragments were excised from the DNA vector with appropriate restriction enzymes and were purified by using a low-melting-temperature agarose gel (16). The cDNA bands were recovered and used as templates in the random-primed DNA labeling technique in the presence of digoxigenin-11-dUTP by using a DNA labeling and detection kit (Boehringer Mannheim, Laval, Québec, Canada).

**Analysis of amplified EAV cDNA and chemiluminescence Southern blot hybridization.** A 10-μl sample of the 100-μl reaction mixture was fractionated by electrophoresis through a 1% agarose gel in TBE buffer (90 mM Tris-borate, 2 mM EDTA [pH 8.0]). The DNA was then visualized under UV light by ethidium bromide (1 μg/ml) fluorescence.

For Southern blot hybridization, the RT-PCR products were transferred onto positively charged nylon membranes and were baked for 1 h at 80°C as described previously (16). The membranes were then treated according to the instructions of the supplier (Boehringer Mannheim). Briefly, the membranes were prehybridized for 2 h at 42°C in the hybridization buffer containing 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0]), 1% Boehringer blocking reagent, 0.2% sodium-*N*-lauroyl sarcosine, 0.02% sodium dodecyl sulfate (SDS), and 50% (vol/vol) formamide. The membranes were hybridized at 42°C for 18 h in fresh hybridization buffer containing 50 pmol of digoxigenin-labeled cDNA probe. Following hybridization, the membranes were rinsed twice at room temperature for 5 min in 2× SSC–0.1% SDS before subjecting them to two final washings of 15 min at 68°C in 0.1× SSC–0.1% SDS. Thereafter, the membranes were subjected to chemiluminescence analysis with a commercial digoxigenin luminescent detection kit (Boehringer Mannheim), which is based on the dephosphorylation by alkaline phosphatase of 3-(2′-spirodamantane)-4-methoxy-4-(3″phosphoryloxy)-phenyl-1,2-dioxetane, disodium salt which leads to a yellow-green light emission. The chemiluminescence-treated membranes were then exposed to X-ray film (X-Omat AR-5; Eastman Kodak Company, Rochester, N.Y.) for various periods of time (usually 5 to 30 min).

## RESULTS

**EAV RNA amplification and cloning of the RT-PCR products.** The primary aim of the work described here was to develop an RT-PCR technique for the detection of EAV in cell culture supernatants and biological samples. However, as a long-term objective, we wished to produce molecular reagents (i.e., nucleic acid probes and recombinant proteins from expression of the entire EAV genes located within the regions of ORFs 3, 4, and 7) to further assist us in our studies of the function of the gene-encoded products and EAV-associated pathogenesis. Therefore, to satisfy both of these objectives, the oligonucleotide primers targeting the entire ORF 3, 4, and 7 sequences, which code for viral structural (ORF 7) or nonstructural (ORFs 3 and 4) proteins, were constructed as shown in Fig. 1A and B. Primers targeting the 3′ end of the polymerase gene (ORF 1b) were selected by using the GENEWORKS primer analysis software (Intelligenetics Inc., Mountain View, Calif.).

Each primer pair amplified a well-defined DNA product of the expected size, as observed on ethidium bromide-stained agarose gels with RNA extracted from an EAV-infected RK-13 cell supernatant (predetermined viral titer of $2 \times 10^6$ $TCID_{50}$s/ml), but not with supernatant or total RNA from control noninfected RK-13 cells or supernatant containing equine infectious anemia virus (2) or equine herpesvirus type I (4), which were used as additional controls. The RT-PCR DNA products were then excised from the agarose gels, cloned into the PCR II TA cloning vector, and sequenced for EAV sequence confirmation. The plasmid constructs were also used as positive control DNA templates in the subsequent RT-PCRs and to generate cDNA probes specific to the 3′ end of

ORF 1b, ORF 3, ORF 4, and ORF 7 by excision of the EAV insert with the appropriate restriction enzyme (*Xba*I, *Hin*dIII, or *Eco*RI).

**Quantitation of the sensitivity of the RT-PCR.** After we demonstrated that the selected oligonucleotide primers and assay conditions were suitable for EAV sequence amplification, we wished to evaluate the sensitivity of the RT-PCR for EAV genome detection. The quantitation assay used was similar to that reported for use in the development of RT-PCRs for genome detection of other RNA viruses (14, 17). Serial 10-fold dilutions were prepared in sterile cell culture medium from the supernatant of EAV-infected RK-13 cell cultures, originally showing a viral titer of $2 \times 10^6$ $TCID_{50}$s/ml. A sample of 100 μl of each viral dilution containing $2 \times 10^5$ to 2 $TCID_{50}$s of EAV was then processed for RNA isolation and RT-PCR as described above. We could amplify the 3′ end of ORF 1b and ORF 4 with as few as 2 $TCID_{50}$s contained in the 100-μl reaction mixture used for RT-PCR, as demonstrated by the presence of DNA bands on the ethidium bromide-stained agarose gel (Fig. 2A and C, lanes 1 to 6). These DNA bands corresponded to the expected size of 535 bp (3′ end of ORF 1b) or 482 bp (ORF 4) on the basis of the published sequence of the EAV genome. To further confirm that the PCR-derived products originated from the EAV genome, the agarose gels were subjected to Southern blot hybridization by using cDNA probes specific to the expected amplified sequences. As shown in Fig. 2B and D, a positive hybridization signal was obtained from each amplification product observed on the corresponding agarose gel, confirming that the DNA fragments amplified by the RT-PCR were homologous to EAV genomic sequences. The hybridization signals observed with the EAV-specific cDNA probes were not observed with the control PCR II TA cloning vector containing no DNA insert or with the proviral DNA of the equine infectious anemia virus that was used as an additional control (data not shown).

When oligonucleotide primers specific to EAV ORF 3 were used in the RT-PCR procedure, only samples containing initial quantities of $2 \times 10^5$ or $2 \times 10^4$ $TCID_{50}$s of the virus produced distinct DNA bands of the expected size (i.e., 494 bp) on the ethidium bromide-stained agarose gel. However, positive signals could be obtained after Southern hybridization with the EAV ORF 3-specific cDNA probe from samples containing as few as 2 $TCID_{50}$s of EAV (Fig. 3A, lanes 1 to 6), showing, by this means, a sensitivity similar to that obtained by amplification of ORF 4 or the 3′ end of ORF 1b.

The use of oligonucleotide primers specific to ORF 7 for EAV genomic amplification resulted in the poorest sensitivity among all primer sets analyzed in the present study. The amplified DNA bands of the expected size (i.e., 342 bp) together with a nonspecific DNA smearing were detected in samples containing only a large quantity ($2 \times 10^4$ $TCID_{50}$s) of EAV particles, even after Southern hybridization (Fig. 3B, lane 2). Although the use of an oligo$(dT)_{12-18}$ primer instead of the sense primer PEV-71 for the synthesis of the first DNA strand and then the DNA amplification step with both primers PEV-70 and PEV-71 allowed the elimination of the nonspecific background smearing, no improvement in EAV ORF 7 detection could be obtained (data not shown). No improvement in EAV ORF 7 detection could be obtained when the reaction mixture was incubated at 95°C for 5 min prior to the RT step or when the solvents dimethyl sulfoxide (21) or formamide (23) were added to the reaction mixture of the amplification step. The "touchdown" PCR approach (11) in the amplification step of the cDNA was also tested, and no improvement in sensitivity was obtained.

The overall ethidium bromide-stained agarose gel and hybridization results obtained from the RT-PCR assays performed with each oligonucleotide primer set are summarized in Table 1 and showed that amplifications of the 3′ end of ORF 1b and ORF 4 gave the best detection levels for the presence of EAV genome of the Bucyrus strain in the cell culture supernatant (Table 1, samples A).

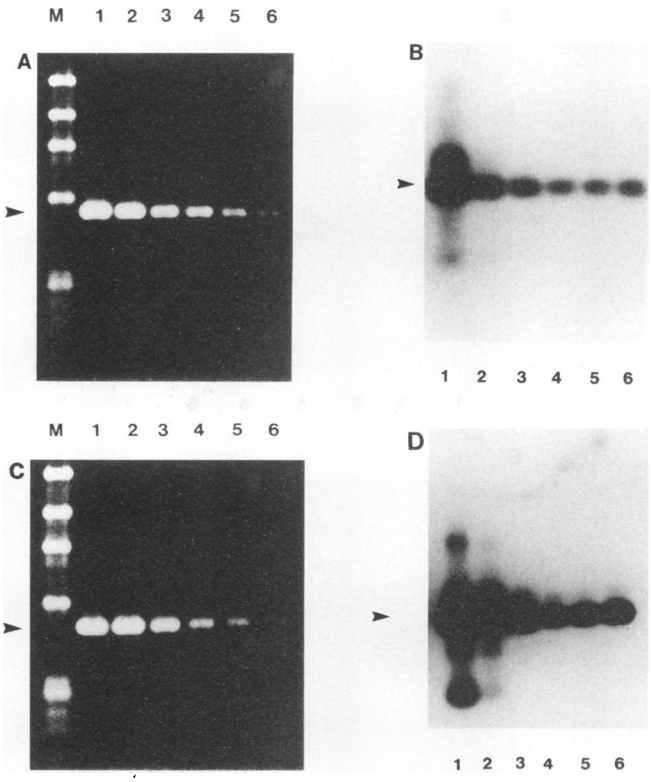



FIG. 2. Sensitivity of RT-PCR for the detection of EAV in cell culture supernatants. The supernatant from the cell culture infected with EAV (starting viral titer of $2 \times 10^6$ $TCID_{50}$s/ml) was serially diluted 10-fold in cell culture medium, and a 100-μl sample of each dilution was processed for RT-PCR. A 10-μl sample of the PCR product was then subjected to gel electrophoresis as described in the text. (A and C) Ethidium bromide-stained agarose gels of the RT-PCR products amplified from the 3′ end of EAV ORF 1b and ORF 4, respectively. (B and D) Chemiluminescence Southern blot hybridization of the agarose gels of panels A and C, respectively, by using EAV cDNA probes specific to each amplified ORF region. Lanes 1 to 6, RT-PCR products obtained from amplification of 100-μl viral mixtures containing $2 \times 10^5$ to 2 $TCID_{50}$s of EAV; lane M, *Hae*III-digested ϕX174 replicative-form DNA as molecular mass markers. The arrowheads mark the positions of the expected products amplified by RT-PCR.

**Detection of EAV genomic RNA in biological samples.** Although no systematic experiment was performed to address the question, we wished to determine whether EAV RNA could be detected in horse semen by RT-PCR. On the basis of the results that were obtained with the EAV-infected cell culture supernatant, we decided to amplify the nucleotide sequences of the 3′ end of ORF 1b and ORF 4 of the EAV genome by using the appropriate primer sets (primers PEV-10 and PEV 11 and primers PEV-40 and PEV-41, respectively). Reconstruction RT-PCR experiments were done with horse semen containing concentrations of $2 \times 10^5$ to 2 $TCID_{50}$s of EAV per 100 μl of sample. As shown in Fig. 4A, a quantity of 20 $TCID_{50}$s (lane 5) of the virus could be detected from the

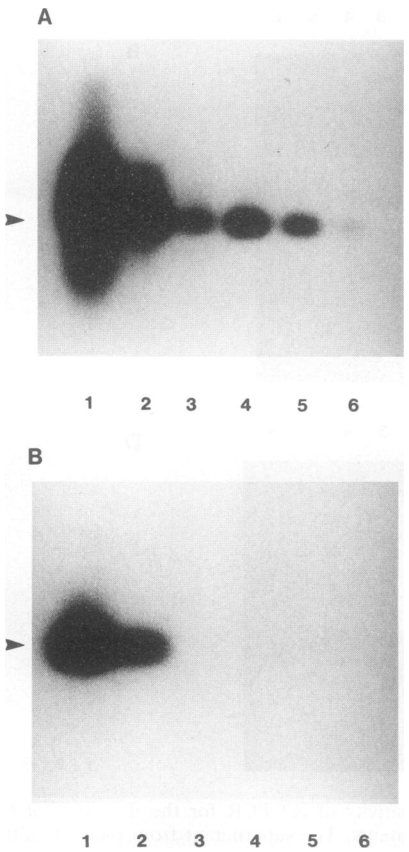



FIG. 3. Chemiluminescence Southern blot hybridization of the RT-PCR products amplified from the EAV ORF 3 (A) and ORF 7 (B) sequences. A cell culture supernatant infected with EAV with a starting viral titer of $2 \times 10^6$ $TCID_{50}$s/ml was serially diluted 10-fold in cell culture medium, and a 100-μl sample of each dilution was processed for RT-PCR. A 10-μl sample of the RT-PCR product was then subjected to gel electrophoresis and, thereafter, was subjected to chemiluminescence Southern blot hybridization with EAV cDNA probes specific to each amplified ORF region. Lanes 1 to 6, RT-PCR products obtained from amplification of a 100-μl viral mixture containing $2 \times 10^5$ to 2 $TCID_{50}$s of EAV. Arrowheads mark the positions of the expected products amplified by RT-PCR.

sample after amplification of the 3′ end of ORF 1b, as determined by the presence of a DNA band (too faint to be reproduced on the photograph) of the expected size on the ethidium bromide-stained agarose gel. Sensitivity was further enhanced to a detection level of 2 $TCID_{50}$s of EAV per sample when the PCR product was subjected to Southern hybridization with the specific cDNA probe (Fig. 4B, lane 6). When the diluted viral samples were amplified with the ORF 4-specific primers, the sensitivity was relatively less than that obtained with the amplification of the 3′ end of ORF 1b, with detection levels of $2 \times 10^2$ $TCID_{50}$s (Fig. 4C, lane 4 [band too faint to be reproduced]) and 20 $TCID_{50}$s (Fig. 4D, lane 5) of EAV, as determined by ethidium bromide-stained agarose gel electrophoresis and Southern hybridization procedures, respectively. The primers specific to the 3′ end of ORF 1b and ORF 4 were then used to test for the presence of EAV by RT-PCR in 16 unknown horse semen samples. As shown in Table 2, 10 samples were found to be positive by the RT-PCR procedure. When this result was compared with that of virus isolation in cell cultures, a concordance of 100% was observed.

TABLE 1. Sensitivity of RT-PCR assay for EAV RNA detection in virus-infected cell culture supernatant and virus-containing horse semen

| ORF target sequence | Sample[a] | Method | Virus detection level ($TCID_{50}$[b]) |
|---|---|---|---|
| 1b, 3′ end | A | Et-Br[c] staining | 2 |
| | A | Hybridization | 2 |
| | B | Et-Br staining | 20 |
| | B | Hybridization | 2 |
| 3 | A | Et-Br staining | $2 \times 10^4$ |
| | A | Hybridization | 2 |
| 4 | A | Et-Br staining | 2 |
| | A | Hybridization | 2 |
| | B | Et-Br staining | $2 \times 10^2$ |
| | B | Hybridization | 20 |
| 7 | A | Et-Br staining | $2 \times 10^4$ |
| | A | Hybridization | $2 \times 10^4$ |

[a] EAV RNA extracted from RK-13 cell culture supernatant (sample A) or semen (sample B).
[b] $TCID_{50}$, median tissue culture infective dose per 100-μl sample.
[c] Et-Br, ethidium bromide-stained agarose gel.

## DISCUSSION

The diagnosis of EAV infection is currently based on either serum antibody determination of virus-exposed animals by complement fixation or virus neutralization tests or virus isolation in cell cultures (3, 12, 13, 24). However, these laboratory procedures are laborious and time-consuming. In the study described here, we tested and used the RT-PCR system for the rapid and sensitive detection of the EAV genome. To do this, the oligonucleotide primers used in the RT-PCR procedure were designed from previously published EAV sequences (7) to amplify the 3′ end of ORF 1b, which encodes the conserved C-terminal extremity of the viral polymerase gene; ORFs 3 and 4, which encode nonstructural proteins to which no function has so far been associated; and ORF 7, which encodes for the nucleocapsid protein. The EAV nucleotide sequences derived from ORFs 2, 5, and 6 were not used as target sequences for the RT-PCR assay because these ORFs encode viral membrane proteins (9) that could possibly be subjected to more frequent genetic variations than the products derived from ORFs 1, 3, 4, and 7, thus contributing to antigenic diversity, as has been reported for some EAV isolates (19).

The results presented here showed that all primer sets used in the RT-PCR procedure targeting the sequences of the ORF 1b 3′ end, ORF 3, ORF 4, and ORF 7 were successful, to different degrees, in amplifying the EAV Bucyrus strain genome. Moreover, we could also amplify sequences from viral RNA obtained from supernatants of four cell culture-adapted EAV field isolates with the same oligonucleotide primers (data not shown). The specificities of the RT-PCR assays were confirmed when the amplified products of the expected size reacted positively with the corresponding virus-specific digoxigenin-labeled cDNA probes in the chemiluminescence assays.

In a previous study (5), detection of the EAV genome by an RT-PCR assay was performed by targeting the leader nucle-

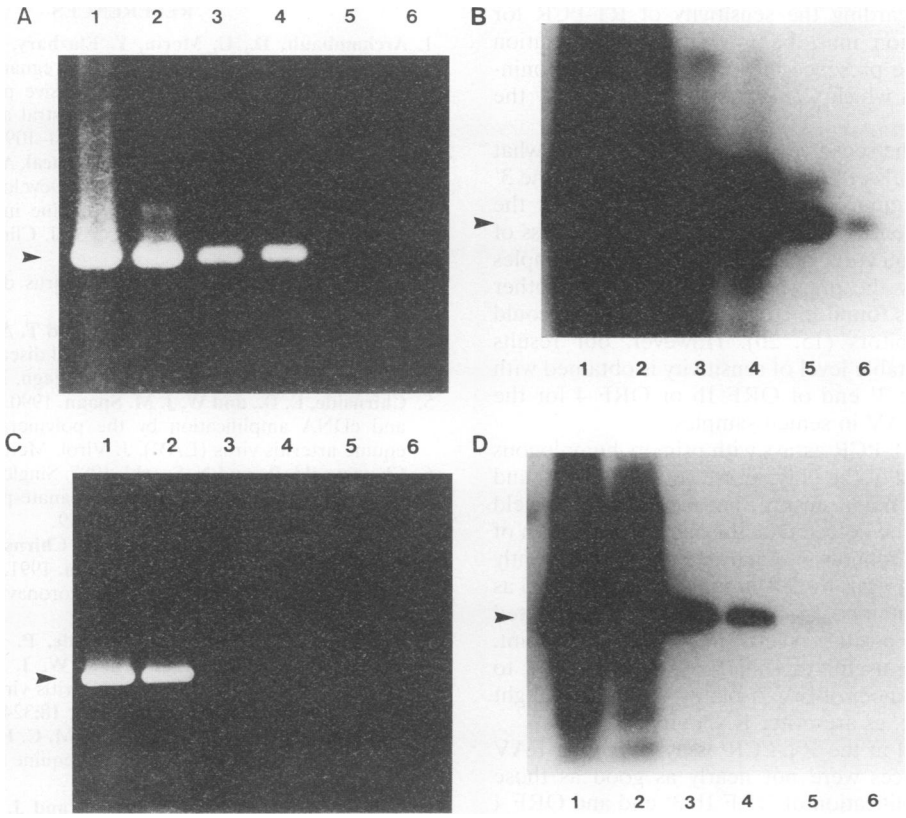



FIG. 4. Sensitivity of RT-PCR for the detection of EAV in horse semen. Normal horse semen to which EAV was added with a resulting viral titer of $2 \times 10^6$ $TCID_{50}$s/ml was serially diluted 10-fold in horse semen, and a 100-μl sample of each dilution was processed for RT-PCR. A 10-μl sample of the RT-PCR product was then subjected to gel electrophoresis as described in the text. (A and C) Ethidium bromide-stained agarose gels of the RT-PCR products amplified from the 3′ end of EAV ORF 1b, and ORF 4, respectively. (B and D) Chemiluminescence Southern blot hybridization of the agarose gels of panels A and C, respectively, by using EAV cDNA probes specific to each amplified ORF region. Lanes 1 to 6, RT-PCR products obtained from amplification of a 100-μl viral mixture containing $2 \times 10^5$ to 2 $TCID_{50}$s of EAV; lane M, *Hae*III-digested ϕX174 replicative-form DNA as molecular mass markers. Arrowheads mark the positions of the expected products amplified by RT-PCR.

otide sequences (nucleotides 15 to 186), the 3′ end of the polymerase gene (ORF 1b; nucleotides 9,246 to 9,524), and the nucleocapsid gene (ORF 7; nucleotides 12,555 to 12,687). However, the primers used by those investigators (5) to amplify the 3′ end of ORF 1b and ORF 7 as well as the expected cDNA fragments obtained in the RT-PCR assays were different from ours. As reported by those authors (5), the RT-PCR procedure performed with primers specific to the leader sequences gave the best results, whereby quantities as low as 600 PFU of EAV per ml could be detected in seminal plasma. By comparison, the sensitivity of the RT-PCR results that we obtained with sets of primers targeting the 3′ end of ORF 1b and ORF 4 sequences appears to be better since we were able to detect as few as 20 or $2 \times 10^2$ $TCID_{50}$s of viral particles, respectively, per 100-μl semen sample, as determined on ethidium bromide-stained agarose gels. The sensitivity of the RT-PCR for the amplification of both targeted sequences increased 10-fold when the agarose gels were subjected to Southern blot hybridization with the EAV-specific cDNA probes.

TABLE 2. Comparison of RT-PCR with virus isolation in cell culture for detection of EAV in semen

| Test | No. of semen samples | | No. of semen samples identical to RT-PCR | % Concordance |
|---|---|---|---|---|
| | Positive | Negative | | |
| RT-PCR[a] | 10 | 6 | NA[b] | NA |
| Virus isolation | 10[c] | 6 | 16 | 100 |

[a] RT-PCR assays were performed with primer pairs targeting the ORF 1b 3′ end and ORF 4 sequences.
[b] NA, not applicable.
[c] The virus infectivity titers of the 10 positive semen samples tested on first passage in RK-13 cells varied from $4.3 \times 10^2$ to $2.6 \times 10^4$ $TCID_{50}$s/ml.

Although all primer pairs used in the present study successfully amplified the cDNA target sequences of the EAV Bucyrus strain as well as those of cell culture-adapted EAV field isolates, the results showed that the degree of sensitivity for EAV detection in infected cell culture supernatants and/or semen samples is variable, depending on the set of primers used. As mentioned above, the primers specific to the 3′ end of ORF 1b and ORF 4 were the most sensitive in detecting EAV cDNA, the ORF 7-specific primers were the least sensitive, and the ORF 3-specific primers were of intermediate sensitivity (Table 1). The RT-PCR performed with sets of oligonucleotide primers targeting the 3′ end of ORF 1b (primers PEV-10 and PEV-11) and ORF 4 (primers PEV-40 and PEV-41) allowed the detection of as few as 2 $TCID_{50}$s of EAV in infected cell culture supernatant, as determined on ethidium bromide-stained agarose gels (Table 1, samples A). However,

the overall results regarding the sensitivity of RT-PCR for EAV genomic detection must be interpreted with caution because of the possible presence in the suspension of noninfectious virus particles which would not be measured by the infectivity assay.

In semen samples, the sensitivity of the assay was somewhat less, with detection levels of 20 $TCID_{50}$s when targeting the 3′ end of the ORF 1b sequence and $2 \times 10^2$ $TCID_{50}$s for the amplified ORF 4 sequences (Table 1, samples B). The loss of sensitivity for EAV detection observed from semen samples might be explained by the presence of polyamines or other unidentified substances found in clinical material which could act as RT-PCR inhibitors (15, 20). However, our results demonstrate that a suitable level of sensitivity is obtained with primers specific to the 3′ end of ORF 1b or ORF 4 for the routine detection of EAV in semen samples.

A combination of RT-PCR assays with primers homologous to the conserved 3′ end of the polymerase gene (ORF 1b) and the ORF 4 sequences might ensure that most if not all field isolates of EAV could be detected by this means. Detection of the EAV genome in unknown semen samples subsequently found to be positive for virus by cell culture isolation as well as the EAV genomes obtained from four cell culture-adapted EAV field isolates are positive results that support our point. However, it might be useful to subject our primer sets to testing with a larger number of EAV isolates, since these might be genetically unstable, as are other RNA viruses (25).

The results obtained in the RT-PCR assays targeting EAV ORF 3 and 7 sequences were not nearly as good as those obtained with the amplification of ORF 1b 3′ end and ORF 4 cDNA sequences. Among the reasons that could explain this poorer detection level is the possible presence of residual inherent secondary structures, even after the initial heating treatment of the sample, near or within genomic regions of the viral RNA that prevent the initial annealing of the antisense primer prior to the RT step and/or impede the RT step itself along the viral RNA template.

The results presented here demonstrate that the RT-PCR procedure, based on amplification of the 3′ end of ORF 1b and ORF 4, can be a useful tool for the diagnosis of EAV and for epidemiologic surveillance. The method is sensitive and has several advantages over conventional tests, including the reliability of the results, a shorter time for the overall procedure, and the lack of a need for aseptic handling. In addition to diagnostic purposes, the molecular tools developed here can be useful for pathogenesis studies associated with EAV infection in the natural host and for gene expression experiments.

## ACKNOWLEDGMENTS

We are grateful to Yolaine D'Astous and Nicole Bellemare for preparation of the manuscript. We thank Denis Vaillancourt and Peter Timoney from the Faculté de Médecine Vétérinaire in St-Hyacinthe, Québec, Canada, and the Gluck Equine Research Center, College of Agriculture, University of Kentucky, Lexington, respectively, for providing the horse semen samples. Peter Timoney also performed the virus titration assays in semen samples. We also thank Susan Carman from the Veterinary Laboratory Services in Guelph, Ontario, Canada, for providing the cell culture-adapted EAV field isolates and Steve Tronick from the Laboratory of Cellular and Molecular Laboratory, National Cancer Institute, Bethesda, Md., for help in designing some of the oligonucleotide primers.

This work was supported by an operating grant from the National Sciences and Engineering Research Council of Canada to D. Archambault. D. Archambault is the holder of a research scholarship from the Fond de la Recherche en Santé du Québec.

## REFERENCES

1. **Archambault, D., G. Morin, Y. Elazhary, R. S. Roy, and J. H. Joncas.** 1988. Immune response of pregnant heifers and cows to bovine rotavirus inoculation and passive protection to rotavirus infection in newborn calves fed colostral antibodies or colostral lymphocytes. Am. J. Vet. Res. **49:**1084–1091.
2. **Archambault, D., Z.-M. Wang, J. C. Lacal, A. Gazit, A. Yaniv, J. E. Dahlberg, and S. R. Tronick.** 1989. Development of an enzyme-linked immunosorbent assay for equine infectious anemia virus detection using recombinant $Pr55^{gag}$. J. Clin. Microbiol. **27:**1167–1173.
3. **Burki, F.** 1965. Eigenschaften des virus der Equinen Arteritis. Pathol. Microbiol. **28:**939–949.
4. **Carman, S., E. Nagy, D. Caldwell, and T. A. Van Dreumel.** 1993. Equine herpesvirus type I neurological disease and interocolitis in mature standarbred horses. J. Vet. Diagn. Invest. **5:**261–265.
5. **Chirnside, E. D., and W. J. M. Spaan.** 1990. Reverse transcription and cDNA amplification by the polymerase chain reaction of equine arteritis virus (EAV). J. Virol. Methods **30:**133–140.
6. **Chomzynski, P., and N. Sacchi.** 1987. Single step method of RNA isolation by acid guanidium thyocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
7. **Den Boon, J. A., E. J. Snijder, E. D. Chirnside, A. A. F. De Vries, M. C. Horzinek, and W. J. M. Spaan.** 1991. Equine arteritis virus is not a togavirus but belongs to the coronaviruslike superfamily. J. Virol. **65:**2910–2920.
8. **De Vries, A. A. F., E. D. Chirnside, P. J. Bredenbeek, L. A. Gravestein, M. C. Horzinek, and W. J. M. Spaan.** 1990. All subgenomic mRNAs of equine arteritis virus contain a common leader sequence. Nucleic Acids Res. **18:**3241–3247.
9. **De Vries, A. A. F., E. D. Chirnside, M. C. Horzinek, and P. J. M. Rottier.** 1992. Structural proteins of equine arteritis virus. J. Virol. **66:**6294–6303.
10. **Doll, E. R., R. E. Knappenberger, and J. T. Bryans.** 1957. An outbreak of abortion caused by the equine arteritis virus. Cornell Vet. **47:**69–75.
11. **Don, R. H., P. T. Cox, B. J. Wainwright, and J. S. Mattick.** "Touchdown" PCR to circumvent spurious priming during gene amplification. Nucleic Acids Res. **19:**4008.
12. **Fukanagara, Y., and W. H. McCollum.** 1977. Complement fixation reactions in equine viral arteritis. Am. J. Vet. Res. **38:**2043–2046.
13. **Golnik, W., Z. Michalska, and T. Michalak.** 1981. Natural viral arteritis in foals. Schweiz. Arch. Tierheilk. **123:**523–533.
14. **Hörling, J., S. Vene, C. Franzen, and B. Niklasson.** 1993. Detection of Ockelbo virus RNA in skin biopsies by polymerase chain reaction. J. Clin. Microbiol. **31:**2004–2009.
15. **Lugaro, G., F. Campagnari, R. Moretti, and M. M. Casellato.** 1988. Inhibition of DNA polymerization and DNA transcription to RNA by seminal plasma peptides. Biochim. Biophys. Acta **950:** 420–428.
16. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
17. **Muir, P., F. Nicholson, M. Jhetam, S. Neogi, and J. E. Banatvala.** 1993. Rapid diagnosis of enterovirus infection by magnetic bead extraction and polymerase chain reaction detection of enterovirus RNA in clinical specimens. J. Clin. Microbiol. **31:**31–38.
18. **Mumford, J. A.** 1985. Preparing for equine arteritis. Equine Vet. J. **17:**6–11.
19. **Murphy, T. W., W. H. McCollum, P. J. Timoney, B. W. Klingeborn, B. Hyllseth, W. Golnik, and B. Erasmus.** 1992. Genomic variability among globally distributed isolates of equine arteritis virus. Vet. Microbiol. **32:**101–115.
20. **Panaccio, M., and A. Lew.** 1991. PCR based diagnosis in the presence of 8% (V/V) blood. Nucleic Acids Res. **19:**1151.
21. **Pomp, D., and J. F. Medrano.** 1991. Organic solvents as facilitators of polymerase chain reaction. BioTechniques **10:**58–59.
22. **Saiki, R. K., S. Scharf, F. Faloona, K. B. Mullis, G. T. Horn, H. A. Erlich, and N. Arnheim.** 1985. Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science **230:**1350–1354.
23. **Sarkar, G., S. Kapelner, and S. S. Sommer.** Formamide can

dramatically improve the specificity of PCR. Nucleic Acids Res. **18:**7465.

24. **Senne, D. A., J. E. Pearson, and E. A. Carbrey.** 1985. Equine viral arteritis: a standard procedure for the virus neutralization test and comparison of results of a proficiency test performed at five laboratories, p. 29–34. *In* Proceedings of the 89th Annual Meeting of the U.S. Animal Health Association, Milwaukee, Wis. U.S. Animal Health Association, Milwaukee, Wis.
25. **Strauss, J. H., and E. G. Strauss.** 1988. Evolution of RNA viruses. Annu. Rev. Microbiol. **42:**657–683.
26. **Timoney, P. J., W. H. McCollum, A. W. Roberts, and T. W. Murphy.** 1986. Demonstration of the carrier state in naturally acquired equine arteritis virus infection in the stallion. Res. Vet. Sci. **41:**279–280.
27. **Westaway, F. G., M. A. Brinton, and S. Y. Gaidamovitch.** 1985. Togaviridae. Intervirology **24:**125–139.