Detection of Equine Arteritis Virus in the Semen of Carrier Stallions by Using a Sensitive Nested PCR Assay

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A nested PCR, developed for the detection of equine arteritis virus (EAV) in semen, detected less than 2.5 PFU of EAV per ml of naturally infected seminal plasma. Based on results from testing 88 semen samples from 70 stallions, the sensitivity and specificity of the test were 100 and 97%, respectively.

Equine arteritis virus (EAV) is the etiological agent of equine viral arteritis, a contagious viral disease of equids, which is characterized principally by fever, anorexia, conjunctivitis, nasal discharge, edema of the limbs and ventral body, and the risk of abortion in pregnant mares (14). Many cases of primary infection with the virus, however, are subclinical in nature. The virus was first isolated from an aborted fetus during an outbreak of respiratory disease and abortion among standardbred horses near Bucyrus, Ohio, in 1953 (6). Transmission of the virus can occur either via the respiratory route or venereally (13).

EAV is the prototype virus of the arteriviruses, a group of small enveloped viruses with positive, single-stranded RNA genomes (10). The arterivirus replication strategy resembles that of the family *Coronaviridae*; namely, a nested set of 3′-coterminal mRNAs with common 5′ leader sequences are produced (3, 4). The genome of EAV is 12.7 kb and consists of seven open reading frames (ORFs) (3). ORFs 2, 5, 6, and 7 encode the four structural proteins of the virus, whereas ORFs 1a and 1b encode the viral polymerase (3, 5).

As high as 30 to 50% of stallions acutely infected with EAV may become persistently infected with the virus. These chronic carriers shed virus continuously in their semen and are responsible for maintenance of EAV in horse populations throughout the world (13). In some stallions, the infection may spontaneously clear after a period of 6 months to a year or longer, whereas in others it may persist indefinitely. Testing semen of seropositive stallions for the presence of EAV is usually required for export purposes and health certification. Traditionally, this has been accomplished by attempted isolation of virus in cell culture or by test breeding a stallion to seronegative mares. Virus isolation (VI) is the more feasible of these two methods for routine testing. Laboratories, however, vary in their abilities to isolate EAV in cell culture on a reliable basis (9). This appears to be due, in part, to the method of VI employed and, in part, to subtle changes which can occur in the susceptibility of the cells to primary infection with this virus (9). Toxicity of semen for cells can also be a problem, as most VI procedures involve inoculation of serial 10-fold dilutions of the sample. While the results from VI may take 2 weeks or more to obtain, a rapid culture method involving antibody staining of viral antigen may yield results in a significantly shorter time (8). Alternatives to the traditional method of VI

are desirable. In this study, we describe a sensitive PCR for the detection of EAV in equine semen which was found to match the sensitivity of a reliable VI procedure.

A total of 88 semen samples collected from 70 different stallions, many of which were known shedders of the virus in semen, were examined by nested PCR and conventional VI. Some 57 of the samples were collected from standardbred horses, 18 were from thoroughbreds, 5 were from Arabians, 2 were from Oldenburgs, and 1 each was from Trakehner, Hanovarian, Tennessee walking horse, Dutch warmblood, Selle Français, and Holsteiner breeds. Multiple samples (two to seven) were tested from nine stallions. All of the standardbred and thoroughbred stallions were of North American origin. Two of the warmblood stallions were known to have been imported from Europe.

Viral RNA was extracted directly from seminal plasma or infectious cell culture medium with TRIzol (Canadian Life Technologies Inc., Burlington, Ontario, Canada) in accordance with a modification of the manufacturer's protocol as described previously (7). Briefly, semen was microcentrifuged at $14,000 \times g$ for 5 min and 20 μg of glycogen and 1 ml of TRIzol were added to 100 μl of collected seminal plasma. After the addition of chloroform (220 μl), the RNA in the aqueous phase (~650 μl) was precipitated with 750 μl of isopropanol for 15 min. The final ethanol-washed RNA pellet was air dried and then dissolved in 50 μl of high-pressure liquid chromatography-grade water.

Attempted VI from seminal plasma was performed as previously described (13). Briefly, confluent monolayers of rabbit kidney (RK-13) cells (ATCC CCL-37) propagated in 25-cm² flasks were inoculated with serial 10-fold dilutions of seminal plasma and overlaid with 0.75% carboxymethyl cellulose. After 5 days of incubation, plaques were visualized by staining the cell monolayer with crystal violet. A second passage was performed if the initial passage showed no cytopathic effect. EAV was confirmed by reduction in titer of the virus with EAV-specific antiserum.

Nested-PCR primers were designed from the published sequence of the ORF1b gene (3), as this gene was expected to be highly conserved among different EAV isolates. The primers were predicted, based on the published sequence, to produce a final product of 186 bp. The external primers, CE (5′ TGGT AGGTGCTTCATTGGCT 3′) and DE (5′ GCGGCACAAG AACACTTCTG 3′), were identical in sequence to the sense and antisense strands from nucleotides 9193 to 9212 and 9610 to 9629, respectively. The internal primers, CI (5′ CCTGAG ACACTGAGTCGCGT 3′) and DI (5′ CCTGATGCCACAT GGAATGA 3′), were identical in sequence to the sense and

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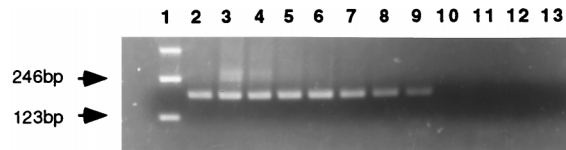
FIG. 1. Detection of EAV in inoculated semen by PCR. RNA was extracted from serial 10-fold dilutions of EAV in seminal plasma and utilized for reverse transcription and PCR. Lane 1 contains a 123-bp ladder (Canadian Life Technologies). Lanes 2 to 12 contain serial 10-fold dilutions, with the sample in lane 2 containing 2×10^7 PFU/ml and the sample in lane 12 containing 0.002 PFU/ml. The detection limit occurs in a sample containing 2 PFU/ml in lane 9. Lane 13 contains negative control semen.

antisense strands from nucleotides 9282 to 9301 and 9447 to 9466, respectively. Primers were designed with Primer Designer for Windows, version 2.0 (Scientific and Educational Software, Stateline, Pa.) and synthesized with an Applied Biosystems model 391 DNA synthesizer.

Two microliters of the extracted RNA was mixed with 5 μM downstream external primer (DE), deoxynucleoside triphosphates (1 mM each; Pharmacia, Baie d'Urfé, Quebec, Canada), 0.4 U of RNAguard RNase inhibitor (Pharmacia) per μl, 200 U of Moloney murine leukemia virus reverse transcriptase (Canadian Life Technologies), 5 mM $MgCl_2$, and reaction buffer (20 mM Tris-HCl [pH 8.4], 50 mM KCl) in a total volume of 20 μl. The mixture was incubated at 37°C for 1 h and then at 95°C for 5 min. First-round PCR reagents, including 2.5 U of *Taq* DNA polymerase (Canadian Life Technologies), were added to the reverse transcriptase mixture to give final concentrations of 1 μM upstream external primer (CE), 2 mM $MgCl_2$, 20 mM Tris-HCl (pH 8.4), and 50 mM KCl in a final volume of 100 μl. After denaturation for 3 min at 94°C, the reaction mixtures were cycled 40 times as follows: 94°C for 20 s, 42°C for 30 s, and 72°C for 30 s with a final extension at 72°C for 15 min. A second round of amplification was performed with 1 μl of the first-round product and the internal primers, CI and DI (1 μM each), by using the same reaction conditions as described for the first round of PCR amplification. The final product (15%) was electrophoresed on a 1.8% agarose gel containing ethidium bromide and photographed under UV transillumination.

The Bucyrus strain of EAV, which was propagated in RK-13 cells, was used to optimize the nested PCR. With cell culture medium and seminal plasma samples to which EAV had been added, a PCR product of ~186 bp consistent with the predicted size was obtained. Based on testing serial 10-fold dilutions of the virus in seminal plasma, the lower limit of detection of nested PCR was found to be ~2 PFU/ml (Fig. 1). The product was confirmed to be specific for the ORF1b gene of EAV by PCR cycle sequencing (data not shown). In addition, no PCR products were obtained from negative control samples or from cell culture samples containing the following equine viruses: equine herpes virus 1 (10^6 PFU/ml), equine herpes virus 4 (2.5×10^7 PFU/ml), equine rhinovirus 1 ($>10^3$ PFU/ml), equine rhinovirus 2 ($>10^3$ PFU/ml), equine influenza virus A1 (hemagglutination titer, 1:128), and equine influenza virus A2 (hemagglutination titer, 1:512).

The results of testing a panel of 36 semen samples by VI and by PCR were in 100% agreement (Table 1). Included in this panel were samples that ranged in virus content from those with very low amounts, i.e., a trace amount (<2.5 PFU/ml) (Fig. 2, lane 13) and 2.5 PFU/ml (Fig. 2, lane 14), to those with high amounts, i.e., up to 130,000 PFU/ml. A prominent PCR product was observed for all samples which were positive by VI.

TABLE 1. Detection of EAV in a panel of 36 semen samples by nested PCR

Sample no.	VI result[a]	PCR result
1	Neg.	−
2	Neg.	−
3	Neg.	−
4	Neg.	−
5	Neg.	−
6	Neg.	−
7	Neg.	−
8	Neg.	−
9	Neg.	−
10	Neg.	−
11	Neg.	−
12	Neg.	−
13	Neg.	−
14	Neg.	−
15	Neg.	−
16	Trace	+
17	2.5	+
18	86	+
19	150	+
20	200	+
21	200	+
22	300	+
23	400	+
24	600	+
25	850	+
26	1,000	+
27	1,700	+
28	2,000	+
29	2,500	+
30	3,000	+
31	8,500	+
32	10,000	+
33	12,000	+
34	15,000	+
35	65,000	+
36	130,000	+

[a] Neg., negative. Amounts of virus detected in culture are reported as PFU per milliliter.

An additional 52 semen samples were tested by VI and PCR; these included 11 samples from four carrier horses that had either spontaneously cleared the infection or else were shedding very low quantities of the virus. The PCR results for these carrier stallions are shown in Fig. 2 (lanes 2 to 12). The semen of stallion A contained 4.5×10^4 PFU/ml of EAV in 1990 (lane 2), while two samples collected in 1994 were negative by VI
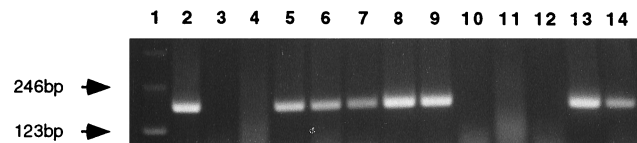

FIG. 2. Detection of EAV in semen samples from carrier stallions by PCR. Lane 1, 123-bp ladder; lanes 2 to 4, results from a spontaneously cleared carrier (horse A), with lane 2 containing 4.5×10^4 PFU/ml, lane 3 containing a subsequent sample (VI negative), and lane 4 containing the sample taken most recently (VI negative); lanes 5 to 9, results from five samples from a low-level-shedding stallion (horse B), with lane 5 containing 40 PFU/ml, lane 6 containing 650 PFU/ml, lane 7 containing 350 PFU/ml, lane 8 containing 1,500 PFU/ml, and lane 9 containing 25 PFU/ml; lanes 10 and 11, results from two successive samples (VI negative) from a spontaneously cleared carrier (horse C); lane 12, result from a spontaneously cleared carrier (horse D) negative by VI; lanes 13 and 14, results from two low-level-shedding stallions, with lane 13 containing trace amounts (<2.5 PFU/ml) of EAV and lane 14 containing 2.5 PFU/ml.

and PCR (lanes 3 and 4). The five semen samples from horse B, a chronic low-grade shedder, were collected over a 2.5-year period. Three positive samples were collected in 1992 (containing 40, 650, and 350 PFU/ml [lanes 5 to 7, respectively]), and one each was collected in 1993 and 1994 (containing 1,500 and 25 PFU/ml [lanes 8 and 9, respectively]). A semen sample from horse C, a spontaneously cleared carrier, was negative by VI and PCR (lane 10) 1 month after a trace positive result was obtained on a sample collected in 1992 (tested by VI only); a subsequent semen sample collected in 1994 was also negative by VI and PCR (lane 11). The semen of stallion D, another spontaneously cleared carrier, was positive for EAV in 1987 (tested by VI only) but negative by VI and PCR for a semen sample collected in 1994 (lane 12). As shown in Fig. 2, the results of the PCR agreed fully with those of VI for these samples.

With respect to the additional 52 samples tested, there were two discordant (positive by PCR and negative by VI) results. One discordant result was apparently due to failure on an initial attempt to isolate EAV, because subsequent retesting of the sample gave a positive result. The second discordant result may have been due to contamination of the sample, as subsequent testing of a second aliquot of the same sample gave negative PCR results. Based on the results of testing 88 semen samples, there was an initial agreement of 98% between the methods. Thus, the diagnostic sensitivity and specificity of the PCR relative to that of VI were 100% (56 of 56) and 97% (31 of 32), respectively. The latter increased to 100% with the repeat PCR result. When both repeat findings were considered, the results of the two methods were in 100% agreement.

Several PCRs for the detection of EAV have been reported (1, 2, 11, 12). Of these, only the nested PCR described by Belak et al. (1) was extensively applied to test semen samples for the presence of EAV. The first EAV PCR, reported by Chirnside and Spaan (2), could detect 600 PFU of EAV per ml in infective semen, whereas the assay reported by St.-Laurent et al. (12) could detect 430 50% tissue culture infective doses per ml of EAV in infective semen. Although the lower limits of detection of virus in semen were not determined for these assays, the PCR of St.-Laurent et al. (12) could detect 20 50% tissue culture infective doses per 100 μl of virus-inoculated semen. The PCR described by Belak et al. (1), which utilized primers designed to amplify nucleocapsid (ORF7) gene sequences, could detect minimal amounts (<1 PFU) of EAV in semen from naturally infected carrier stallions.

The nested PCR described herein, which uses primers based on ORF1b sequences, was highly sensitive. It yielded a product from a semen sample in which EAV occurred in trace amounts, producing plaques only on second passage in RK-13 cells. Considering that the recommended minimal dilution of semen tested is 1:10 in order to circumvent cellular toxicity, the lowest quantity of virus that can be routinely determined by the VI method used in this study was 2.5 PFU/ml (i.e., 1 plaque per four inoculated flasks). Thus, the lower limit of detection of the PCR described herein can be expressed as <0.25 PFU or <2.5 PFU/ml. This high level of sensitivity suggests that the PCR used in this study can effectively deal with any PCR-inhibiting

substances in semen (12). The advantage of this PCR, over that of other PCRs for detection of EAV in semen, is that it combines rapid processing of samples (i.e., shorter RNA extraction times) with high sensitivity.

The results of this study support the use of the PCR as an alternative method to VI for the detection of EAV in semen. Initial results with homogenates of infected liver and lung samples (not shown) suggest that the PCR may also have general applicability for the detection of EAV in other biological materials. Although the PCR cannot determine the infectivity of EAV in equine semen, the results indicate that it can be used to determine whether equine semen is free of EAV. This is an important requirement in the standard examination of stallions for health certification purposes. The rapidity with which PCR results can be obtained (2 days) is a great advantage over conventional VI procedures, especially in facilitating the international movement of horses.

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