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Detection of Equine Infectious Anemia Viral RNA in Plasma Samples from Recently Infected and Long-Term Inapparent Carrier Animals by PCR†

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Control of equine infectious anemia (EIA) is currently based on detection of anti-EIA virus (EIAV) antibodies. However, serologic diagnostic methods may give false-negative results in infected horses that fail to respond adequately or are in the early stages of infection. We developed a reverse transcriptase nested PCR (RT-nPCR) assay for the detection of viral *gag* gene sequences in plasma from EIAV-infected horses. The ability of RT-nPCR to detect field strains of EIAV was investigated by assaying plasma samples from 71 horses stabled on EIA quarantine ranches. Positive PCR signals were detected in 63 of 63 horses with EIAV antibody test-positive histories on approved serologic tests, demonstrating that RT-nPCR was probably directed against highly conserved sequences in the viral genome. The RT-nPCR assay, agar gel immunodiffusion test, and conventional virus isolation were compared for detection of early infection in 12 experimentally infected ponies. Viral *gag* sequences were detected in all 12 animals by 3 days postinfection (p.i.) by RT-nPCR, whereas virus could not be routinely isolated on cell culture until 9 to 13 days p.i. and EIAV antibodies could not be detected by agar gel immunodiffusion until 20 to 23 days p.i. Finally, specificity of the RT-nPCR assay was examined by testing plasma from 43 horses with serologic test-negative histories and no known contact with EIAV-infected animals. Viral *gag* sequences were not detectable in this control group. These data suggest that the EIAV RT-nPCR assay effectively detects EIAV and is more sensitive than current standard methods for detection of early stages of infection.

Horses that survive the acute and chronic episodes of equine infectious anemia (EIA) often progress to an inapparent stage in which no overt signs of disease are evident. Despite the lack of clinical signs, these horses still represent a permanent economic loss to the equine industry and a source of infection to other horses (26). Control of EIA is based on identification of inapparent carriers by detection of antibodies to EIA virus (EIAV) in serologic tests, generally the agar gel immunodiffusion (AGID) test (9) or enzyme-linked immunosorbent assay (ELISA)-based tests (6, 33). The current internationally accepted standard for diagnosis of EIAV infection is the AGID, which detects antibodies to the major *gag* gene product, p26. The p26 antigen is the most abundant protein in virus preparations and contains group-specific determinants (13, 45). Competitive ELISA (CELISA) also detects antibodies to the p26 antigen and has been approved for official use in the United States because correlation of AGID and CELISA results is excellent (33, 47). The synthetic antigen ELISA detects antibodies to the gp45 antigen (6). The diagnostic efficacy of these assays has been well established, but some indeterminate results still occur. Currently, all ELISA-positive samples require confirmation by AGID before regulatory controls are imposed. In confirmatory diagnoses of human immunodeficiency virus (HIV) infections, routinely applied tests include the Western blot (immunoblot assay) (6, 31) and direct detection of viral nucleic acids in plasma or whole blood by either the branched DNA hybridization technique (5, 42) or PCR analysis (2, 41). Similar confirmatory tests have yet to be fully developed or evaluated in EIAV infections.

The immunoblot test has given researchers a method to confirm anti-EIAV antibody reactivity from sera that exhibit equivocal AGID or CELISA reactions (30, 49). Immunoblot testing detects antibodies to the envelope glycoproteins gp90 and gp45, in addition to those directed against p26. The Centers for Disease Control and Prevention criterion for a positive immunoblot test for the related lentivirus HIV is the presence of antibodies to any two of p24, gp41, and gp120/160 (7, 16). Alternatively, the Food and Drug Administration criterion for a positive HIV immunoblot test is the presence of antibodies to p24, p31, and one envelope glycoprotein (15). Any banding patterns not meeting Centers for Disease Control and Prevention or Food and Drug Administration criteria are regarded as an indeterminate result, while only samples presenting no bands are considered negative. No such guidelines have been established for interpretation of EIAV immunoblot testing.

All serologic tests for EIAV have recognized limitations. The principal drawback of serodiagnostics is that these tests evaluate presence of a host immune response and not specifically the presence of plasma viremia. Besides individuals in the initial stages of EIAV infection that have immature humoral responses, horses that appear unable to generate antibody responses to EIAV have been documented (24, 27, 30, 34, 56). In both situations, the presence of low quantities of anti-

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p26 antibodies can be falsely interpreted as negative in the officially accepted diagnostic tests (30). Another potential drawback is the presence of antibodies directed against the interspecies determinant of the p26 antigen (21, 36). These antibodies could arise in the horse following exposure to related lentiviruses in nature. Serum samples that are positive in both AGID and CELISA but react with only p26 in the immunoblot test and not with the immunodominant antigen, gp90 or gp45, have been tested (23a). These limitations of serodiagnostic procedures and documented existence of "seronegative" carriers warrant investigation of more definitive and practical tests for the presence of EIAV (24, 35, 56).

Direct detection of EIAV is desirable for several reasons. Development of a direct assay would help in identification of individuals who are infected but are tested before seroconversion. Detection of neonatal EIAV infection would be simplified, because maternal antibodies to EIAV can occasionally persist beyond 6 months of age, and passive maternal and active immunity cannot be differentiated by AGID or CELISA (25). Direct detection of virus would permit clarification of the infection status of individuals with indeterminate serologic reactions. A direct assay would also provide a means to monitor viral burdens in active infections. This study was undertaken to identify a definitive and practical test for detection of EIAV in plasma from horses.

MATERIALS AND METHODS

Animal subjects. Experiments were carried out under the regulations and guiding principles of the University of Kentucky Institutional Animal Care and Use Committee. Ponies ($n = 12$) were infected with $10^{2.5}$ tissue culture infectious doses of the pathogenic variant of EIAV derived from the cell-adapted strain of EIAV (28). Whole blood from each pony was sampled before infection and from 2 days to 36 months postinfection (p.i.). Horses (Thoroughbred, Standardbred, quarter horse, and mixed breed) ($n = 71$) stabled on three EIA quarantine ranches were each sampled once. Another group of repeatedly test-negative horses ($n = 43$) with no known exposure to EIAV-infected horses was also sampled for use as negative controls.

Plasma sample preparation. Whole blood was sampled by venipuncture into blood collection tubes containing an acid-citrate dextrose anticoagulant (Becton Dickinson Vacutainer Systems; Becton Dickinson, Rutherford, N.J.). Samples were held on ice between the time of sampling and that of further processing. Blood samples were processed within 1 h of collection. Whole blood was centrifuged ($400 \times g$ for 10 min) to harvest plasma that was immediately stored at −70°C.

EIAV serology. Serum samples were collected on the same occasions as plasma. Samples were tested for antibodies to the p26 antigen of EIAV by AGID (46) and CELISA (IDEXX Laboratories Inc., Westbrook, Maine) (57), by standard protocols. Antibodies to other structural viral polypeptides were examined by immunoblot analyses as described previously with the cell-adapted strain of EIAV as antigen (50), except that TMB (3,3′,5,5′-tetra-methyl-benzidene; Promega, Madison, Wis.) was used as a substrate for the horseradish peroxidase-conjugated, anti-horse immunoglobulin G antibody.

Virus isolation and detection. Techniques for the maintenance of primary fetal equine kidney cell cultures and virus isolation from plasma samples have been described previously (28). Growth of EIAV in cell cultures was monitored by a microassay for reverse transcriptase (RT) activity (10).

RNA isolation from plasma samples. Total RNA was extracted from plasma samples by low-speed centrifugation, as described previously (8, 59), with the following modifications. Briefly, a 2-ml aliquot of plasma was placed in a 2.2-ml microcentrifuge tube and centrifuged ($14{,}000 \times g$, 30 min, 10°C). The supernatant was aspirated, and the pellet was dissolved in 400 μl of a solution containing 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol. The following reagents were added to resuspended pellets: 1 μg of *Saccharomyces cerevisiae* tRNA in 10 μl of diethyl pyrocarbonate-treated water, 26 μl of 3 M sodium acetate (pH 4.5), 400 μl of Tris-saturated phenol (pH 8.0), and 80 μl of chloroform-isoamyl alcohol (49:1). Samples were vortexed thoroughly, placed on ice for 20 min, and then centrifuged ($12{,}500 \times g$, 20 min, 4°C). The upper aqueous phase was removed, and 400 μl of isopropanol was added. This solution was vortexed and placed at −20°C for 2 to 24 h. The RNA was then precipitated by low-speed centrifugation ($12{,}500 \times g$, 20 min, 4°C). The resulting pellet was dissolved in 200 μl of a solution containing 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol and reprecipitated with 200 μl of isopropanol. The RNA pellet was washed with successive additions of 1, 0.5, and 0.2 ml of 75% ethanol. Air-dried pellets were resuspended in 10 μl of RNase-free diethyl pyrocarbonate-treated water and stored at −80°C. The entire sample was then subjected to RT-nested PCR (RT-nPCR). Samples extracted by this method and nPCR amplified without an RT step gave negative results, demonstrating that those RT-nPCR positives obtained were from EIAV cDNA and not from EIAV proviral DNA contamination (data not shown).

TABLE 1. Oligonucleotide primers used in the RT-nPCR assay

Primer	Sequence (5′ to 3′)	Position in EIAV genome[a]
p26-1	GGCATCATTCCAGCTCCTAAGA	*gag* p15 758–779
p26-2	ATGTTTGTGCTGCCTTTAGTGG	*gag* p11 1589–1611
p26-11	CAGGCAGGACAAAAGCAGATA	*gag* p26 1023–1043
p26-22	GCTTTAGGTTTTCCAATCATCAC	*gag* p26 1262–1285

[a] Genomic positions based on published sequences of the cell culture-adapted Wyoming strain of EIAV (GenBank accession numbers for EIAVCG: M16575, K03334, M11337, and M14855).

Primers. An RT-nPCR assay was designed with primers based on nucleotide sequence information (55) obtained from the cell culture-adapted Wyoming strain of EIAV (32). Nucleotide sequences and relative positions of the synthetic oligonucleotide primers used in the RT-nPCR assay are shown in Table 1. Primers were synthesized by standard phosphoramidite chemistry with a Model 380B DNA Synthesizer (Applied Biosystems, Foster City, Calif.) at the Macromolecular Structure Analysis Facility, University of Kentucky, Lexington.

Reverse transcription. Reverse transcription of EIAV RNA was carried out under mineral oil (Sigma, St. Louis, Mo.) in an adjusted total volume of 20 μl, containing 10 μl of RNA sample, in 50 mM KCl–10 mM Tris-HCl (pH 8.3)–4.5 mM $MgCl_2$–1 mM (each) deoxynucleoside triphosphates, 1 U of RNase inhibitor (Perkin-Elmer Cetus, Norwalk, Conn.)–2.5 U of Moloney murine leukemia virus RT–0.75 μM p26-2 primer (Table 1). The reaction mixture was incubated at 42°C for 45 min before being heated to 99°C for 5 min. An automated thermocycler (Model 480; Perkin-Elmer Cetus) was used for all incubations.

nPCR amplification. Following reverse transcription, 80 μl of a solution containing 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 U of *Ampli Taq* DNA polymerase (Perkin-Elmer Cetus), and 0.75 μM p26-1 primer (Table 1) was added to each reaction mixture. An amplification profile consisted of 30 cycles for 30 s at 94°C for denaturation, 30 s at 50°C during the first 5 cycles and 45°C during the other 25 cycles for the annealing temperature, and 30 s at 72°C for elongation. The final extension period was 7 min at 72°C, followed by an incubation at 4°C. An aliquot (5 μl) of this first PCR was added to the second nPCR mixture containing 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1 mM deoxynucleoside triphosphates, 0.75 μM primer p26-11, 0.75 μM primer p26-22 (Table 1), and 2.5 U of *Ampli Taq* DNA polymerase in a final volume of 100 μl. Reaction mixtures were overlaid with mineral oil, and the same amplification profile was used as described above. Amplification products were analyzed by agarose gel electrophoresis (0.8% agarose; Tris-borate buffer [pH 8.0]) and visualized by ethidium bromide staining. The presence of a DNA fragment of the expected sizes (853 bp on first stage, 262 bp on second stage) was determined by migration of the PCR product in comparison with a 100-bp DNA double-stranded ladder (Gibco-BRL) and positive control standards. The identity of the DNA fragment was confirmed as the *gag* gene region of EIAV by Southern blot hybridization (51, 54).

RNA standard synthesis. A region in the EIAV *gag* gene thought to be highly conserved among divergent viral isolates was selected as the target sequence. A 1,547-bp *Bam*HI-*Xba*I fragment encompassing the entire major core protein (p26) was subcloned into a pGEM-3Zf(+/−) vector (Promega) and placed under the control of a T7 promoter to allow generation of in vitro transcripts for use as positive controls in RT-nPCR. The transcripts were prepared as runoff products of the DNA template and linearized at a *Hin*dIII site located just 3′ to the EIAV sequence insert (final transcript size was 1,597 bp). In vitro transcription was performed with T7 polymerase and a commercially available kit (Promega). Standard RNA was transcribed from 5 μg of the construct DNA at 37°C for 2 h (100-μl volumes of 40 mM Tris-HCl [pH 7.5], 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol; 0.5 mM (each) ATP, GTP, CTP, and UTP; 100 U of RNasin, 30 U of T7 RNA polymerase). Transcribed cRNA was treated with RQ1 RNase-free DNase (Promega), purified by phenol-chloroform extraction and ethanol precipitation, resuspended in diethyl pyrocarbonate-treated water, and quantitated by spectrophotometry. The number of cRNA copies was calculated on the basis of spectrophotometry at 260 nm and the molecular size of the transcript (1,597 bp). cRNA was portioned into single-use aliquots and stored at −70°C. Dilutions of cRNA were prepared immediately before inclusion in each RT-nPCR assay.

Assay standardization. (i) Efficiency of RNA recovery. Plasma samples from a horse with no known contact with EIAV-infected animals and repeatedly test negative on AGID and CELISA were spiked with known amounts of *gag* cRNA transcripts and organically extracted to determine the efficiency of RNA isolation. Triplicate sets of samples spiked with 87.5 fg, 8.75 fg, 0.875 fg, 87.5 ag, 43.8 ag, 8.75 ag, 4.38 ag, and 0.875 ag of *gag* cRNA transcripts corresponding to 10^5,

TABLE 2. Sensitivity of nested PCR

Stage	Signal at copy no.[a]:						
	10^4	10^3	10^2	50	10	5	1
First	+	+	+/−	−	−	−	−
Second	+	+	+	+	+	+	+/−

[a] +, presence of signal; +/−, variable presence of a signal; −, no detectable signal.

10^4, 10^3, 10^2, 50, 10, 5, and 1 copies, respectively, were processed as described above.

(ii) Efficiency of reverse transcription. Reverse transcription reaction efficiency was measured by RNA transcription from the construct pGEM-3Zf(+/−), containing a T7 promoter region and a 1,547-bp fragment that encompassed the entire *gag* coding region (final transcript size was 1,597 bp). The *gag* cRNA was transcribed as described above. The concentrations of *gag* cRNA transcripts used for subsequent cDNA synthesis and *gag* plasmid DNA used for quantitative comparison were estimated by spectrophotometry at 260 nm. Twofold serial dilutions of cDNA after reverse transcription with reverse primer (p26-2) and 10-fold dilutions of *gag* plasmid DNA were made before PCR amplification with *gag*-specific primers (p26-1, p26-2). The PCR amplifications were done as described above, and the resulting PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining. cDNA synthesized was estimated by reference to a dilution series of *gag* DNA after amplification with the same primers. This was then compared with the number of cRNA transcripts from which the cDNA was synthesized.

(iii) Optimization of PCR parameters. Optimal reaction conditions were established for detection of EIAV cRNA by PCR with *gag*-specific primers (Table 1). The RT-nPCR cycling profile described above was used to determine the requirements for $MgCl_2$ concentrations in the amplification reaction mixtures. Optimal reaction conditions were biased in favor of maximal amplification of the expected-size PCR product.

(iv) Sensitivity of nPCR. The sensitivity of the nPCR test was determined by using a dilution series of cloned *gag* DNA as targets. Triplicate sets of first-stage reaction mixtures were set up with dilutions reaching 0.52 pg, 52 fg, 5.2 fg, 0.52 fg, 0.26 fg, 52 ag, 26 ag, and 5.2 ag of *gag* plasmid DNA that corresponded to 10^5, 10^4, 10^3, 10^2, 50, 10, 5, and 1 copies, respectively. First- and second-stage reaction amplification products were analyzed by agarose gel electrophoresis and ethidium bromide staining.

RESULTS

Assay standardization. (i) Efficiency of cRNA recovery. Efficiency of viral cRNA recovery from plasma samples was established by performing the extraction procedure on 2-ml aliquots of plasma from an EIAV-negative animal seeded with predetermined log dilution copy numbers of a *gag* cRNA transcript. The extraction procedure was repeated three times at each copy number level. The number of cRNA copies recovered was calculated on the basis of spectrophotometry and molecular size of the transcript. Efficiency of viral cRNA recovery was estimated to be 65% at the lowest levels of detection (data not shown).

(ii) Efficiency of reverse transcription. RT reaction efficiency was estimated with cRNA transcribed from a plasmid containing *gag* gene sequences. A known amount of cRNA transcripts from the cloned *gag* gene was reverse transcribed, and the cDNA was serially diluted before PCR amplification with the *gag*-specific primers p26-1 and p26-2 (Table 1). This was compared with the results of amplifying a log dilution series of cloned *gag* DNA with the same primers. The cutoff point, below which amplified DNA was not detectable by agarose gel electrophoresis and ethidium bromide staining, was 5.2 fg for the *gag* plasmid DNA. By using the known molecular size of the *gag* plasmid (4,746 bp), this number corresponded to 5×10^2 molecules of *gag* plasmid DNA, or approximately 10^3 copies of target sequence (500×2 for double-stranded DNA). The cDNA synthesized from 10 pg of an EIAV *gag* cRNA transcript was serially diluted before amplification. A dilution containing cDNA synthesized from 10 fg of cRNA gave a positive result, and the next dilution containing cDNA from 5.0 fg was negative. With the estimated molecular size of the RNA transcript (1,597 bp), the minimum detectable amount of cDNA corresponded to an input of 1.14×10^4 molecules of cRNA. Efficiency of the RT reaction, in terms of molecules of amplifiable cDNA synthesized from cRNA templates, is the ratio of the cutoff point of *gag* copy number detection to the minimum detectable amount of cDNA ($10^3/1.14 \times 10^4$), or approximately 8.8%.

(iii) Optimization of PCR parameters. The influence of $MgCl_2$ concentrations on the detection sensitivity of *gag* viral gene sequences at decreasing amounts of target DNA was investigated. Four different $MgCl_2$ concentrations (1, 1.5, 2, and 2.5 mM) were studied with four *gag* plasmid DNA amounts (5, 1, 0.5, and 0.1 ng). All these amplifications were carried out with the cycling profile described above, and the resulting products were analyzed by agarose gel electrophoresis with ethidium bromide staining. From these results (data not shown), the optimal reaction conditions were as follows: 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1 mM deoxynucleoside triphosphates, and 0.75 μM primers. These conditions were selected on the basis of maximal amplification of the expected-size fragment and the absence of secondary coamplification products.

(iv) Sensitivity of nPCR. Sensitivity of the nPCR was determined by the addition of known amounts of *gag* plasmid DNA to reaction mixtures. The nPCR assay was repeated three times at each copy number level. The cutoff point, below which first-stage PCR products were not consistently detectable by agarose gel electrophoresis and ethidium bromide staining, was 8.37 fg or 10^3 copies of target sequence. In second-stage reactions, products of the predicted size could be consistently detected following the addition of five copies (41.84 ag) of the *gag* plasmid DNA substrate (Table 2). Both first- and second-stage products were confirmed to be complementary to the EIAV *gag* gene nucleotide sequence by Southern hybridization (data not shown) (51, 54).

Prospective studies of EIAV-infected horses. (i) Early diagnosis in experimentally infected ponies. Comparisons of RT-nPCR, conventional virus isolation techniques, and the detection of anti-EIAV antibodies for the early diagnosis of EIAV were performed with 12 ponies experimentally infected with $10^{2.5}$ tissue culture infectious doses of a pathogenic variant of the cell culture-adapted strain of EIAV. Samples from each pony were tested before infection and shown to be negative in

TABLE 3. Correlation of RT-nPCR, virus isolation, and serologic testing of 12 ponies experimentally infected with the pathogenic variant strain of EIAV[a]

Pony no.	First plasma RT-nPCR signal detected	First positive virus isolation	First positive AGID reaction
1	2	11	21
06	3	13	21
11	2	13	22
33	2	13	21
35	3	12	21
49	3	11	21
72	3	11	24
80	3	13	25
85	3	5	21
86	3	10	21
87	3	13	21
98	3	9	20

[a] Values shown are days p.i.

TABLE 4. Serologic and RT-nPCR results of horses stabled on EIA test-positive and EIA test-negative farms

No. of animals	Age[a]	Diagnostic history	AGID[b]	CELISA[b]	Western[c] (26/45/90)	RT-nPCR[d] (1st/2nd)
43	3 to 25	Positive 1973 to 1994[e]	+	+	+/+/+	+/+
20	5 to 25	Positive 1974 to 1993	+	+	+/+/+	−/+
2	16	Neg (1993)[f]	−	−	+/−/−	+/+
	21	Neg (1992)				
1	28	Neg (1993)	−	−	+/−/−	−/+
2	23	Neg (1994)	−	−	−/−/+	−/+
	29	Neg (1979)				
1	21	Neg (1986)	−	−	−/−/−	−/+
2	NA[g]	NA	−	−	−/−/−	−/−
	17	Neg (1992)				
43	1	Neg (1992)	−	−	−/−/−	−/−

[a] Years of age on date of sampling.
[b] Positive and negative test results are shown by plus and minus signs, respectively.
[c] Specific protein bands of reactivity for the Western (immunoblot) assay are p26, gp45, and gp90 and are shown as 26/45/90. +, positive; −, negative.
[d] Signals on the first or second stage of RT-nPCR are shown as 1st/2nd. +, positive; −, negative.
[e] Year of first positive serologic test result.
[f] Negative results on all official serologic tests since the date given.
[g] NA, not available.

both AGID and RT-nPCR. All 12 ponies produced a positive signal in RT-nPCR by 3 days p.i. (Table 3). Furthermore, positive signals were also produced by RT-nPCR in each of 49 follow-up plasma samples taken from these ponies over a period of 36 months (data not shown). By contrast, in only one case (pony 85, Table 3) could the virus be isolated on cell culture as early as 5 days p.i. In all other cases (11 of 12), virus was not isolated until 9 to 13 days p.i. Serum samples from each pony were generally positive in immunoblot tests at 14 days p.i. (data not shown), with the first positive AGID being detected 20 to 25 days p.i. (Table 3).

(ii) False-positive reaction controls. Plasma samples from 43 horses that had no known contacts with EIAV-infected animals and were repeatedly test negative on AGID and CELISA were tested in RT-nPCR. No PCR amplification products were detected from these animals (data not shown). In addition, EIAV RNA samples amplified without RT also gave negative nPCR results (data not shown). This shows that the positive signals obtained in nPCR were from EIAV cDNA, not from EIAV proviral DNA contamination.

(iii) Natural infection study. Results of field studies are shown in Table 4. Seventy-one horses (Thoroughbred, Standardbred, quarter horse, and mixed breed) from three EIA quarantine ranches were bled and tested for EIAV infection. Serum samples were tested by AGID, CELISA, and immunoblot analysis. All 63 horses with serologic test-positive histories were positive by AGID and CELISA and had antibodies against the EIAV proteins gp90, gp45, and p26 as determined by immunoblot analysis. The 63 horses also had detectable EIAV *gag* gene sequences by plasma RT-nPCR assay. Of these, 43 (68%) had EIAV detectable on first-stage PCR, and 20 (32%) were detectable only on the second stage.

Eight of the 71 horses had test-negative histories despite being pastured with test-positive horses for as long as 20 years. All eight were test negative in AGID and CELISA at the time of sampling. Five of these eight horses had detectable immunoblot reactions to one EIAV protein and were RT-nPCR positive. Three of these eight horses had antibodies against p26 as determined by immunoblot analysis, one had EIAV sequences detectable on first-stage PCR, and two had EIAV sequences detectable on second-stage PCR. The other two horses had antibodies against gp90 as determined by immunoblot analysis and had EIAV sequences detectable on second-stage PCR. The remaining three horses were negative by all serologic procedures, and yet one had EIAV sequences detectable on second-stage PCR.

DISCUSSION

Conventional serodiagnostic assays for detection of anti-EIAV antibodies are convenient and useful for large-scale population screening, but these tests have significant limitations. The most obvious limitation is an inability to detect EIAV during early stages of infection. Anti-EIAV antibodies are either absent during this period or present in amounts undetectable by AGID or either of the two approved ELISA formats. Nonetheless, these animals can serve as important sources for transmission of EIAV because plasma viremia is usually present. An additional limitation of AGID is demonstrated by the observation that horses infected with EIAV, as confirmed by horse inoculation test, may have equivocal reactions in AGID (24, 27, 30, 34, 56). Sensitive immunoblot tests have been a valuable asset in clarifying the infection status of horses with indeterminate reactions in official EIA tests (27). However, the immunoblot test is also subject to indeterminate reactions (27). The Food and Drug Administration and Centers for Disease Control and Prevention guidelines for diagnosis of HIV infections require reactivity with at least two of the major structural antigens of HIV, whereas immunoblot reactivity with only a single major structural antigen of EIAV has been identified in EIAV-infected horses (27). Data presented here suggest that RT-nPCR can be used to elucidate the status of horses with indeterminate reactions on serologic tests and identify EIAV infection before seroconversion.

Useful diagnostic tests require reproducibility, high sensitivity, and high specificity. We have investigated several factors that affect the utility of the EIAV RT-nPCR assay as a diagnostic test. These included the efficiency of RNA isolation, reverse transcription efficiency, and PCR sensitivity and specificity. Sequence design of amplification primers is the primary factor governing the sensitivity and specificity of PCR assays (18). Specificity of RT-nPCR in this study was achieved by using a specific set of internal primers designed from a gene region conserved among known EIAV isolates and demonstrated not to cross-react with equine cellular DNA. In addition, GenBank and EMBL database nucleotide sequence comparisons (1) revealed no homology to any deposited sequences. To obtain the sensitivity required for detection of EIAV RNA in clinical plasma samples, an nPCR protocol was adopted. A detection limit of between one and five copies of *gag* target sequence was achieved by the nPCR assay. If the test is envisaged to diagnose clinical infection rather than to quantify an infectious virus, restriction of amplification to viral RNA is unnecessary (23). In this study, no DNase treatment was performed before RT-nPCR. However, reactions performed without RT showed that insufficient EIAV proviral DNA was present in plasma specimens for detectable amplification.

Reproducibility and specificity studies show that the EIAV plasma RT-nPCR assay was accurate, showing no reactivity with samples from 43 horses that were repeatedly test negative on AGID or CELISA and had no known exposure to EIAV-infected horses. Agreement of test results among plasma RT-nPCR, virus isolation, and serodiagnostics was evaluated during experimental infections. Studies of 12 experimentally

infected ponies showed that direct detection of EIAV by RT-nPCR had greater sensitivity early in infection than AGID, virus isolation, or immunoblot analysis. Positive plasma RT-nPCR signals (mean = 2.8 days) were detected before virus isolation (mean = 11.2 days) or positive AGID (mean = 21.6 days) was observed. This significant advantage over the current serodiagnostic tests suggests that RT-nPCR could be useful in field situations in which recent exposure is suspected.

The RT-nPCR primers were designed on the basis of the published nucleotide sequence for the cell culture-adapted strain of EIAV (55). Despite the presence of these sequences in all previously described EIAV isolates, it was not known whether they were extensively conserved in field isolates of EIAV. Therefore, to investigate the efficacy of the RT-nPCR in naturally infected animals, 71 horses stabled on EIA quarantine ranches were sampled and tested for EIAV infection. Sixty-three of these horses had serologic test-positive histories and were positive on AGID and CELISA at the time of sampling. These horses also had detectable antibodies against the EIAV proteins p26, gp45, and gp90 in immunoblot analyses and were RT-nPCR positive. The RT-nPCR results showed that 43 (68%) of these horses were positive on first-stage PCR. Efficiency studies showed that the minimum detectable quantity of viral RNA in 2 ml of plasma on first-stage PCR was 5,000 copies, suggesting that these animals had at least 5,000 circulating viral RNA molecules per 2 ml of plasma. The other 20 (32%) RT-nPCR-positive horses were positive only on second-stage PCR, suggesting that these animals had between 5 and 5,000 detectable copies of circulating viral RNA. No apparent correlation between time of first positive AGID and high or low viral RNA copy number was identified.

The remaining eight animals had serologic test-negative histories despite being pastured with the other test-positive horses for up to 20 years. All eight were test negative in AGID and CELISA at the time of sampling. Two of these horses had antibodies only against gp90 as determined by immunoblot analysis, and both had EIAV *gag* gene sequences detectable on second-stage PCR. The presence of antibodies to gp90 in the apparent absence of those to p26 could be explained by exposure to cross-reactive epitopes from the major envelope glycoproteins of other retroviruses. However, this is unlikely because EIAV *gag* gene sequences were detected by RT-nPCR in these animals. Instead, these observations could result from a failure of individual horses to respond to *gag* antigens or to the potent antigenic properties of gp90 that normally induce antibody titers 10 to 100 times greater than those to p26 (40, 45). These immunogenic properties could account for the fact that, following infection with EIAV, antibodies to gp90 are usually detected before those to p26 and that only antibodies to gp90 can be detected in situations in which there are extremely low levels of viral replication. This was observed in ponies transfected with DNA from an infectious molecular clone of EIAV containing a deletion in the dUTPase gene (11). Therefore, horses having detectable antibodies to gp90 but not to p26 may be either in the acute stage of EIAV infection or infected with a strain of the virus with an inherently low rate of replication. Both questions could be resolved in an appropriately designed prospective study.

Even more problematical results were obtained from three of the eight remaining horses that were negative in both AGID and CELISA but reacted with p26 in immunoblots. Although the seroreactivity to p26 could represent nonspecific reactions, this is unlikely as EIAV *gag* gene sequences were detected by either first-stage ($n = 1$) or second-stage ($n = 2$) PCR. Therefore, antibodies to p26 were probably present in these horses at concentrations too low for detection by the relatively insensitive AGID or CELISA format. However, if this assertion proved correct, lack of reactivity with the viral glycoproteins would have to be reconciled with the above statements concerning the highly immunogenic nature of gp90.

It is of course possible that these horses were exposed to some related retrovirus. The *gag* gene protein determinants are conserved among different viral isolates and between retroviruses in general, probably indicative of intrinsic properties of the protein that are vital to virus function (4, 17, 19–21, 37, 39). Specifically, many studies have detailed the cross-reactivities between the major core protein of HIV and the analogous protein of EIAV (21, 22, 36, 52), simian immunodeficiency virus (38), and bovine immunodeficiency-like virus (20, 58). Serological cross-reactions between lentiviruses are probably indicative of immune system recognition of highly conserved conformational epitopes among these phylogenetically related retroviruses (22). However, lack of homology between known retroviruses and the RT-nPCR primers suggests that, if these horses had been exposed to some related retrovirus, it is one that has not been previously described. Although such viruses may await discovery, it is also possible that some horses may have defects in T-helper or B-cell responses to the glycoproteins of EIAV. Additional explanations could include infection with a defective strain of EIAV (although presumably this would require some form of a helper virus to be perpetuated) or infection with a strain of EIAV that has almost no homology with the *env* genes of known isolates of this virus. Clearly, additional studies will be required to resolve these questions. These investigations could include the use of PCR to amplify other regions of the viral genome in these horses followed by comparative nucleotide sequence analyses.

The remaining three horses were negative by all serologic procedures, and yet one had EIAV detectable on second-stage PCR. This may represent an individual that was in the early stage of infection (before days 15 to 60) (29), similar to the finding of RT-nPCR-positive ponies early in experimental infection. Alternatively, this finding of a positive RT-nPCR could represent a false-positive test. Carryover of PCR products and cross-contamination of samples within the laboratory are a particular problem with highly sensitive tests such as PCR (53). However, all assays in this study were performed in tandem with multiple reagent controls, to which no DNA template was added. Therefore, the EIAV RNA detected in the plasma of this serologic test-negative horse is likely to represent the true infection status.

Evaluations of new diagnostic assays that are more sensitive than existing techniques can be difficult. First, discrepancies will inevitably arise between the old test and the newer, more sensitive assays. Use of the existing test as the standard for determining absolute infection status may cause a falsely low specificity for the new, more sensitive test. Ideally, additional information such as the use of longitudinal samples should be used to resolve these discrepancies.

An important implication of our findings would be that direct virus detection from plasma by RT-nPCR should be preferred for the diagnosis of EIAV infection in individuals with indeterminate test results. However, RT-nPCR assays may be subject to variability in RT and PCR efficiencies and could be affected by the stability of samples during transport and storage. The effects of plasma sample processing and storage conditions on the recovery of HIV type 1 (HIV-1) RNA have been investigated previously (12, 59). These studies show that HIV-1 RNA is not measurably degraded upon repeated freeze-thawing of plasma or after several days of storage at room temperature (12). Similar results have been reported for the stability of hepatitis C virus RNA in plasma (57). Additional studies

have shown no difference in HIV-1 RNA recovery rates from plasma stored for up to 12 months with or without the chaotrope guanidine thiocyanate, compared with plasma samples assayed immediately after collection (59). However, use of RT-nPCR as a standard field test is probably impractical since control of EIAV is dependent on routine wide-scale testing to identify and control movement of infected horses.

These preliminary studies of experimentally and naturally infected horses show that direct detection of EIAV by RT-nPCR assay has greater sensitivity in identification of horses infected with EIAV than either AGID or CELISA. Plasma RT-nPCR could play a key role in diagnosis of EIAV infection in cases with indeterminate serodiagnostic test results or under conditions in which determination of the absolute infection status of a horse is necessary. The RT-nPCR can be employed to identify EIAV in experimentally infected horses, days (mean = 8.4 days) before virus isolation assays and nearly 3 weeks (mean = 18.8 days) before they become antibody test positive in AGID or CELISA. Negative plasma RT-nPCR results could also be used to identify false-positive serologic reactions. Detection of EIAV antibody and plasma RNA can also be used to confirm EIAV infection in individuals with equivocal serologic results associated with early seroconversion or late-stage disease.

This study also demonstrated that EIAV RNA can be detected in plasma throughout infection. The EIAV RNA could be detected in experimentally infected ponies before seroconversion and in all seropositive horses tested, including animals infected for more than 20 years. Similar results have been published for other retrovirus infections (3, 14). Most HIV-1-seropositive individuals have between 10^2 and 10^7 virions per ml of plasma, irrespective of the clinical stage (43, 44, 48). However, for humans infected with HIV-1, or sheep infected with visna/maedi virus and goats infected with caprine arthritis-encephalitis virus, persistent retroviral replication results in a disease state that becomes progressively debilitating. In contrast, horses infected with EIAV often become inapparent carrier animals with no overt clinical signs. These differences cannot be explained by EIAV becoming latent in the classic sense of the term. Data presented here, along with demonstrations that infectious virus can be transmitted by inapparent carriers (25), suggest that EIAV is probably actively transcribed throughout the lifetime of the infected horse. Furthermore, estimates of viral copy number in long-term EIAV-infected horses may be in the same order of magnitude as that found in some HIV-1 patients (43, 44, 48). If this is confirmed by additional studies, it would suggest that the immune system of the horse does not provide freedom from disease simply by exerting greater control over retroviral replication compared with the immune systems of other species. Further investigation of this phenomenon could lead to an understanding of the mechanism by which the horse and virus coexist and lead to further understanding of HIV pathogenesis.

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