Identification of Noncytopathic Equine Rhinovirus 1 as a Cause of Acute Febrile Respiratory Disease in Horses

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Equine rhinovirus 1 (ERhV1) is a recognized cause of acute febrile respiratory disease in horses, although the virus is rarely isolated from such animals, despite seroprevalence rates as high as 50% in some horse populations. Recently, ERhV1 has been shown to be most closely related to foot-and-mouth disease virus, raising questions as to its disease associations in horses. We report that ERhV1 infection was the likely cause of two separate outbreaks of severe febrile respiratory disease which involved more than 20 horses. Attempts to isolate ERhV1 from nasopharyngeal swabs by conventional cell culture methods were unsuccessful, in that cytopathology was not observed. Viral antigen was detected by immunofluorescence assay in the cytoplasm of cells infected with 10 of 15 nasopharyngeal swab samples, indicating the presence and presumably replication of ERhV1. A rise in serum neutralizing antibody titer between acute- and convalescent-phase sera confirmed that ERhV1 was causatively associated with one of the outbreaks. ERhV1 RNA was detected in nasopharyngeal swabs collected from all horses during the acute phase of disease by reverse transcription-PCR. Nucleotide sequencing of amplified products showed that within each outbreak a single strain of ERhV1 was involved but that distinct viruses were involved in each outbreak. A retrospective study of samples from nine other outbreaks of respiratory disease in horses suggested ERhV1 etiology in at least two of these. We conclude that the relative importance of ERhV1 as a cause of acute febrile respiratory disease in horses has been underestimated due to failure in many instances to isolate virus by conventional cell culture methods.

Equine rhinovirus 1 (ERhV1) infection occurs worldwide (2, 6, 7, 15, 18) and is a recognized cause of an acute respiratory disease with systemic clinical signs in horses. Disease is characterized by fever, anorexia, nasal discharge, coughing, pharyngitis, and lymphadenitis of the head and neck. Infection is also accompanied by viremia and long-term fecal and urinary shedding of the virus (10, 14). The incidence of neutralizing antibody to ERhV1 has been shown to vary according to the age of the horse. In a study by Studdert and Gleeson (17), 16% of horses between 6 and 12 months of age were seropositive, rising up to 53% in some populations comprising horses more than 12 months old (17). Despite this, ERhV1 is relatively rarely isolated, and hence rarely diagnosed, as the cause of disease. Also, it has been our experience that the vast majority of outbreaks of febrile respiratory disease in horses, in cases in which a viral etiology appeared likely, go undiagnosed.

Based on biophysical properties, particularly acid lability, ERhV1 was named and tentatively included in the genus *Rhinovirus* in the family *Picornaviridae* (14). Subsequent studies (11, 12, 17) indicated that several properties, which included nucleic acid density and base composition, a broad host range both in cell culture and in animals, and an apparent lack of extensive antigenic variation, ERhV1 differed from other members of the *Rhinovirus* genus. It was also notable that the diseases produced by ERhV1 included not only respiratory signs but systemic signs that included high fever and viremia. The investigators suggested that ERhV1 may be more closely related to the foot-and-mouth disease viruses, which comprise the *Aphthovirus* genus, than to members of the *Rhinovirus* genus (12, 17). Determination of the complete sequence of the

polyprotein and some of the flanking sequences of the ERhV1 genome (8, 21) confirmed that ERhV1 is most closely related to members of the *Aphthovirus* genus. Although it may be considered a distant member of the *Aphthovirus* genus, some distinctive genomic features and biological properties led to the proposal that ERhV1 may most usefully be considered a member of a new genus.

In this report we show that noncytopathic strains of ERhV1 were causatively associated with two outbreaks of acute febrile respiratory disease in which more than 20 horses were affected. Evidence which implicates ERhV1 as the likely cause of at least two other separate outbreaks of respiratory disease in horses is also presented.

MATERIALS AND METHODS

Clinical samples. Nasopharyngeal swabs and some sera were collected from three sets of horses. Samples in sets 1 and 2 were obtained from horses involved in two outbreaks of acute febrile respiratory disease. Set 1 samples were obtained from 10 broodmares (samples 1.1 to 1.10) on a thoroughbred breeding farm. Each of the mares showed clinical signs that included fever, anorexia, copious nasal discharge, and severe pharyngitis. Acute- and convalescent-phase sera were also obtained from six of these mares during a 41-day period beginning from the acute phase of disease. Approximately 1 year after the outbreak, nasopharyngeal swabs were collected from 2 of the original 10 mares (samples 1.12 and 1.13, corresponding to samples 1.3 and 1.6, respectively) and also from 4, apparently healthy, unrelated horses (samples 1.11, 1.14, 1.15, and 1.16) on the same farm. Samples in set 2 (samples 2.1 to 2.10) were from 10 of 14 2- to 3-year-old thoroughbred horses that had been assembled in a single stable complex prior to international transportation. Each of these horses had an acute febrile respiratory illness characterized by nasal discharge. Sera were not collected from any of these horses. In both outbreaks, other horses from which samples were not obtained had similar clinical signs. Samples in set 3 (samples 3.1 to 3.10) comprised 10 nasopharyngeal swabs that were collected from horses involved in nine separate outbreaks of acute respiratory diseases between 1993 and 1996 in Australia from which virus was not isolated in cell culture.

Cells and viruses. Equine fetal kidney (EFK) monolayer cell cultures at the fifth passage and the Vero cell line of African green monkey kidney at passage 220 were used for virus cultivation. The isolation and nucleotide sequence of

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ERhV1.393/76 have been described previously, and this strain was used as a positive control in both PCR and serological assays (8, 17, 18). ERhV1.967/90 was isolated on EFK monolayer cell cultures from a nasopharyngeal swab from a horse, which had been imported to Australia 3 months earlier from Argentina, during an outbreak of acute respiratory disease (4). This strain was included in the analyses as a positive control for indirect immunofluorescence assays (IFAs) and PCR as well as for the purpose of VP1 sequence comparison.

Virus isolation from clinical samples was performed as described previously (17). Inoculated cell cultures were incubated for 5 to 7 days. Infected cell cultures were then frozen-thawed prior to passaging three times on EFK or Vero monolayer cell cultures. Samples passaged three times in EFK cells were passaged a further time in Vero cells. Filtrates of 10 samples from set 1 were also inoculated into the allantoic cavities of 10-day-old specific-pathogen-free (SPF) embryonated chicken eggs (SPF Poultry Unit, Commonwealth Scientific and Industrial Research Organisation, Victoria, Australia).

IFAs. Vero monolayer cell cultures were grown in the wells of Lab-Tek chamber slides (Nunc Inc., Naperville, Ill.). Filtrates from nasopharyngeal swabs (100 µl) were inoculated onto Lab-Tek chamber slides, and the slides were incubated for 1 h at 37°C. The inoculum was then removed and replaced with culture medium (minimum essential medium with nonessential amino acids and supplemented with 1% fetal bovine serum, 0.13 M NaHCO$_3$, 0.015 M N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid [HEPES; pH 7.5], and 50 µg of ampicillin per ml). After 4 days of incubation at 37°C, the wells were washed twice with phosphate-buffered saline, fixed in methanol for 30 min, and dried at room temperature. The slides were incubated for 30 min at 4°C with rabbit antiserum raised to ERhV1.393/76 antigen (17) or normal rabbit serum diluted 1:10 in phosphate-buffered saline containing 2.5 mg of bovine serum albumin per ml. Bound antibody was detected with biotinylated swine anti-rabbit immunoglobulin diluted 1:250 (Dako, Hamburg, Germany), followed by a 1:500 dilution of avidin-fluorescein conjugate (Pierce, Rockford, Ill.). The slides were examined by immunofluorescence microscopy and photographed (Zeiss Axioskop microscope).

Oligonucleotide primers. Synthetic oligonucleotide primers (DNagency, Aston, Pa.) based on the ERhV1.393/76 nucleotide sequence (8) were the VP1 genome sense primer (VP1F; 5′-GTTGTGTTCAAGATGCAGGC-3′), the 2A genome antisense primer (R2A; 5′-TTGCTCTCAACATCTCCAGC-3′), and the VP1 genome antisense primer (VP1R; 5′-TAGCACCCTCCTTTATCATGCG-3′) (see Fig. 2).

RNA extraction, RT, and PCR. Virion RNA was extracted from nasopharyngeal swabs (600 µl) and from infected cell culture lysates by using RNeasy Total RNA kits (Qiagen, Germany), and the RNA extract was resuspended in a final volume of 26 µl.

For reverse transcription (RT), 26 µl of RNA diluted in diethyl pyrocarbonate-treated water was incubated at 37°C for 60 min in the presence of RT buffer (50 mM Tris-HCl [pH 8.3], 50 mM KCl, 10 mM MgCl$_2$, 10 mM dithiothreitol, 0.5 mM spermidine [Promega, Madison, Wis.], 3 mM [each] deoxynucleotide triphosphates [New England Biolabs, Beverly, Mass.], 30 U of human placental RNase inhibitor [RNA-guard; Pharmacia, Uppsala, Sweden], 100 pmol of R2A primer, and 25 U of avian myeloblastosis virus reverse transcriptase [Promega]) in a total volume of 40 µl.

For the first round of nested PCR amplification, 4 µl of the RT mixture was added to 96 µl of PCR mixture which contained thermophilic buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.6], 0.1% Triton X-100 [Promega]), 0.2 mM (each) deoxynucleotide triphosphate (New England Biolabs), 100 pmol (each) of primers of R2A and VP1F, and 0.5 U of *Thermus aquaticus* DNA polymerase (Promega). Amplification consisted of one round of denaturation at 95°C for 5 min followed by 35 cycles of 30 s at 95°C, 30 s at 60°C, and 45 s at 72°C. For the seminested PCR, 4 µl of the first PCR mixture was added to 96 µl of a second-round PCR mixture which was identical to the first one except that primer R2A was replaced with the internal primer VP1R. Ten microliters of the PCR mixtures was separated by electrophoresis in 2.5% agarose gels in Tris-phosphate buffer and was stained with ethidium bromide (16). Southern blotting and hybridization were carried out as described elsewhere (16). To obtain a VP1 probe, plasmids containing ERhV1.393/76 VP1 (8) and ERhV1.967/90 (9) were pooled and labelled with [α-^{32}P]dCTP (specific activity, 3,000 Ci/mmol; Amersham) by using a random primer labelling kit (Boehringer, Mannheim, Germany).

DNA cloning and sequencing. PCR products were extracted with phenol-chloroform and subsequently purified through MicroSpin S-400 HR columns (Pharmacia), blunt ended with the Klenow enzyme in the presence of deoxynucleotide triphosphates, and phosphorylated by using T4 polynucleotide kinase (Boehringer). The products were ligated into the *Sma*I site of pUC18 (Pharmacia). Ligated products were transformed into *Escherichia coli* DH5α (Stratagene, La Jolla, Calif.) by electroporation. Two colonies containing the insert from each PCR product were sequenced by dideoxy chain termination (16) with modified T7 DNA polymerase (Pharmacia) and [^{35}S]dATP (Amersham). Sequencing products were separated in 6% polyacrylamide wedge gels. The sequence was read and analyzed with the GeneWorks software package (Intelligenetics, Mountain View, Calif.). GenBank database searches were performed by using the FASTA searching and comparison program (13). Nucleotide and protein sequence alignments were performed by using the ClustalW software package (19) or the comparison program in the Genetics Computer Group software package (5).

SN test. Sera were assayed for the presence of ERhV1-specific neutralizing antibody by standard serum neutralization (SN) tests carried out in a microtiter system as described previously (17). Twofold dilutions of serum were mixed with an equal volume of 100 50% tissue culture infective doses of ERhV1.393/76. Serum-virus mixtures were incubated for 60 min at 37°C before the addition of cells. Microtiter plates were incubated at 37°C in a humidified incubator with 5% CO$_2$. Following 7 days of incubation, the wells were examined for cytopathic effects (CPEs) and the SN antibody titer was calculated.

RESULTS

Diagnosis of ERhV1 infection in horses involved in two outbreaks of acute febrile respiratory disease. Nasopharyngeal swabs were obtained from 10 horses involved in each of two separate outbreaks of acute febrile respiratory disease. Attempts were made to isolate virus by standard cell culture methods by passaging three times in EFK or Vero monolayer cell cultures. Samples passaged in EFK cells were passaged a further time in Vero cells. At no stage was a CPE observed for any of the samples. No virus was grown in embryonated chicken eggs.

Filtrates from the original samples inoculated onto Vero monolayer cell cultures were tested by IFA for evidence of replicating ERhV1 by using a rabbit serum raised to ERhV1.393/76. Cells infected with ERhV1.967/90 displayed a strong cytoplasmic fluorescence when probed with the ERhV1 antiserum (Fig. 1). Immunofluorescence was observed in the cytoplasms of Vero cells inoculated with 2 of 5 primary samples from set 1 and 8 of 10 samples from set 2 (Fig. 1; Table 1), indicating the presence of replicating ERhV1 in these cells. No immunofluorescence was observed in Vero cells inoculated with a nasopharyngeal swab collected from an SPF foal infected with equine herpesvirus 2 (3) or in cells infected with ERhV1.967/90 and incubated with normal rabbit serum (Fig. 1).

An RT-PCR was developed and was used to detect the presence of ERhV1 RNA in the clinical samples. The PCR was designed to amplify a 362-bp region at the 3′ end of the VP1 gene in a first-round reaction and a 212-bp fragment in a nested second-round reaction (Fig. 2). First-round product was detected following ethidium bromide staining in 8 of 10 samples from horses in set 1 and 4 of 10 samples from horses in set 2 (data not shown). Second-round PCR product was amplified and was visualized in each of the 20 samples by ethidium bromide staining (Fig. 3A and C). The specificities of the products, both first- and second-round products, was confirmed by Southern blotting and hybridization to a VP1 probe (Fig. 3B and D). In the case of ERhV1.967/90, the predominance of the first-round product was probably due to poor amplification of the second-round product as a result of nucleotide differences in the VP1R primer binding site (see Fig. 4A).

Nasopharyngeal swabs were obtained approximately 1 year after the outbreak from 2 of the original 10 mares from set 1 (samples 1.12 and 1.13), as well as from 4 horses from the same property that were clinically unaffected by the outbreak of respiratory disease (samples 1.11 and 1.14 to 1.16). Evidence of ERhV1 replication and virus-specific antigen synthesis was not found by IFA in Vero cells inoculated with nasopharyngeal swab filtrates (Table 1). By contrast to the primary samples obtained during the acute phase of the disease (samples 1.1 to 1.10), first-round or second-round PCR products were not detected in any of the secondary samples by ethidium bromide staining (Fig. 3A). However, second-round PCR products were detected following Southern blotting and hybridization to a VP1 probe in samples from the two original mares (samples 1.12 and 1.13 are secondary samples obtained from the same horses from which primary samples 1.3 and 1.6, respectively,
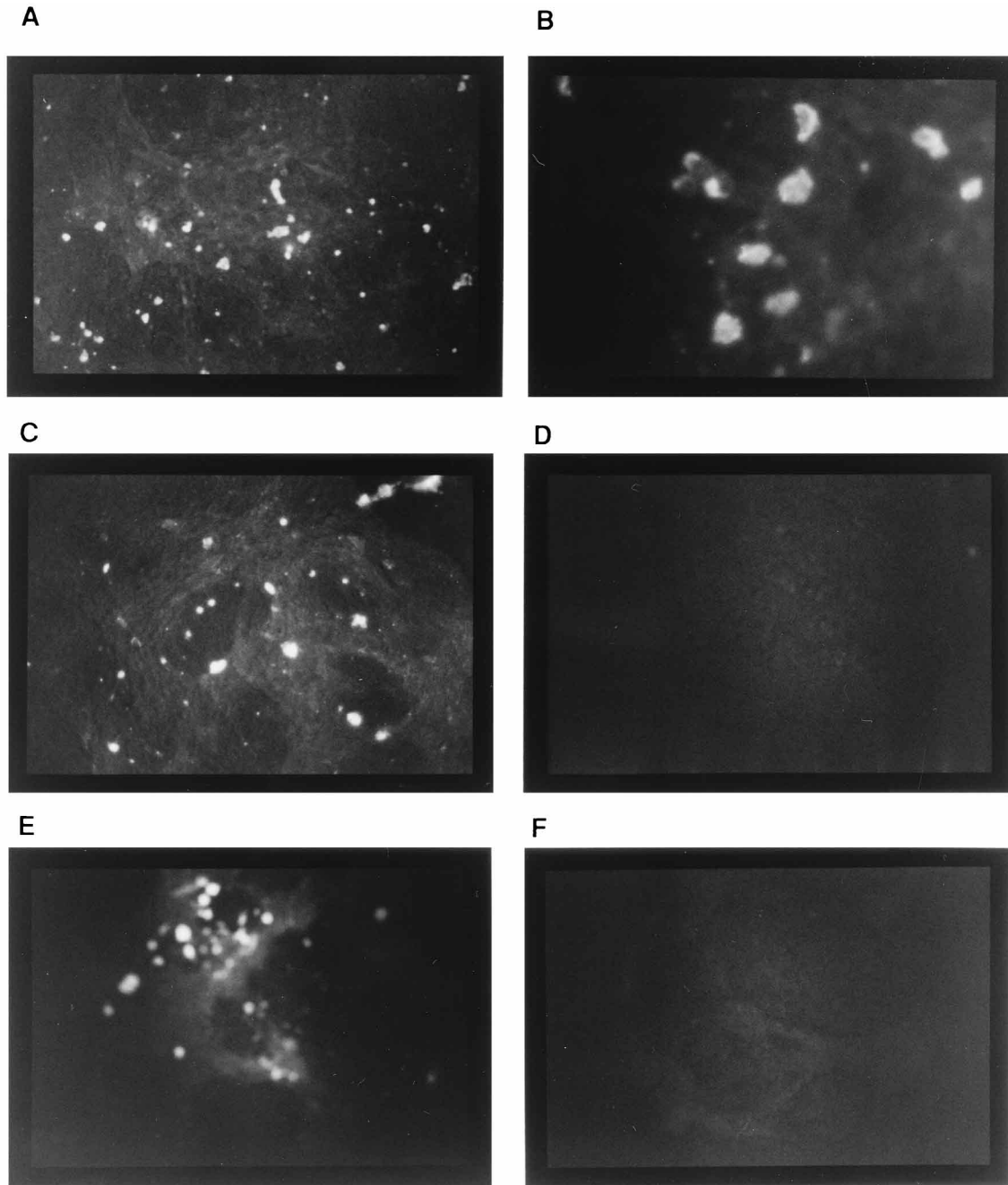
FIG. 1. Indirect immunofluorescence assays of representative samples for the detection of ERhV1 antigens in Vero monolayer cell cultures. Filtrates of nasopharyngeal swabs were inoculated. (A) Sample 1.4 (from a mare; magnification, ×160); (B) sample 1.4 (from a mare; magnification, ×400); (C) sample 2.1 (from a mare; magnification, ×200); (D) ERhV1.967/90 (magnification, ×200); (E) ERhV1.967/90 (magnification, ×200); (F) sample from an SPF foal experimentally infected with EHV2 (magnification, ×200). Cells shown in panels A, B, C, E, and F were incubated with rabbit anti-ERhV1.393/67 serum, while those in panel D were stained with normal rabbit serum.

were obtained) and from one of the other four mares (sample 1.15; Fig. 3B), although these bands were faint in comparison to the bands representing second-round products collected from acute-phase samples.

Serum samples were also obtained from six horses (samples 1.1 to 1.6) over a 41-day period beginning from the acute phase of disease (day 0). Acute-phase sera were only available for two horses (samples 1.1 and 1.2), and both of these were seronegative. Serum collected from these two horses at day 20

showed 200- and 400-fold increases in ERhV1 neutralizing antibody, respectively (Table 2). While acute-phase sera were unavailable for four horses (samples 1.3 to 1.6), each showed near or above a fourfold rise in serum neutralizing antibody titer between days 5 and 41 (Table 2).

Nucleotide sequencing of RT-PCR-amplified VP1 cDNA. First-round PCR products, comprising the 3′ region of the VP1 gene, which were amplified from RNA extracted from nasopharyngeal swabs collected from four horses (samples 1.4, 1.7,

TABLE 1. Detection of ERhV1 antigen by indirect
immunofluorescence assay

Sample no. or source	ERhV1 antigen[a]
1.1	ND[b]
1.2	−
1.3	+
1.4	+
1.5	ND
1.6	ND
1.7	−
1.8	−
1.9	ND
1.10	ND
1.11	−
1.12	−
1.13	−
1.14	−
1.15	−
1.16	−
SPF foal[c]	−
ERhV1.967/90	+
2.1	+
2.2	+
2.3	−
2.4	+
2.5	+
2.6	−
2.7	+
2.8	+
2.9	+
2.10	+

[a] ERhV1 antigen was detected with rabbit anti-ERhV1.393/76 serum.
[b] ND, not determined.
[c] From an SPF foal infected with equine herpesvirus type 2.

2.1, and 2.7), were cloned and the nucleotide sequences were determined. The two VP1 sequences amplified from within each sample set were identical (data not shown), which was consistent with the fact that each outbreak was caused by the particular ERhV1 strain represented by the sequence and was unlikely to be due to persistent virus in the respiratory tracts of these horses. However, the VP1 sequence from set 1 (designated ERhV1.1090/94) was considerably different from that obtained from set 2 (designated ERhV1.1165/95), which indicated that the two outbreaks were epidemiologically unrelated (Fig. 4A) as well as geographically distinct (1,500 km).

The nucleotide sequence of ERhV1.967/90 VP1 was also determined, and the three sequences were aligned with the corresponding region of ERhV1.393/76 VP1 (Fig. 4A). Exten-

sive nucleotide sequence differences were observed between the four sequences, with 95 of the 300 bases being variable. When translated into amino acid sequences, 15 amino acid differences between the four sequences were apparent, with the majority of the 95 nucleotide substitutions being silent. Twelve of the variable amino acid positions were localized to two regions of VP1 between amino acids 155 and 158 and amino acids 209 and 217.

Retrospective analyses of clinical samples from horses with acute febrile respiratory disease. Ten nasopharyngeal swabs were collected from horses involved in nine separate outbreaks of acute, febrile respiratory disease. Attempts to isolate virus from each of these samples in EFK monolayer cell cultures were unsuccessful (data not shown). Samples were examined for the presence of ERhV1 RNA by RT-PCR. Following first-round PCR only samples 3.3, 3.5, and 3.6 yielded a product which was visible by ethidium bromide staining. After hybridization to a VP1 probe, ERhV1 cDNA could be detected in samples 3.1, 3.3, 3.5, 3.6, and 3.9. In the second-round PCR, only samples 3.1, 3.5, and 3.9 yielded a product visible with ethidium bromide staining, and the specificities of these products were confirmed by DNA hybridization (data not shown). The lack of amplification of ERhV1 in samples 3.3 and 3.6 in the second-round PCR suggests sequence variation in the VP1R primer binding site, similar to that observed for strain ERhV1.967/90, and suggests a common strain of ERhV1 consistent with an ERhV1 etiology in this outbreak.

DISCUSSION

We describe two outbreaks of respiratory disease in thoroughbred horses that were caused by ERhV1 but in which cytopathogenic virus could not be isolated. A high degree of confidence was placed in the diagnosis of ERhV1 disease in the outbreak represented by sample set 1 in which evidence of ERhV1 infection was adduced by RT-PCR, by replication of ERhV1 in cell culture as detected by IFA, and from seroconversion based on serum neutralization assays with acute- and convalescent-phase sera. In the case of the second outbreak, represented by sample set 2, diagnosis of ERhV1 infection was based on the results of RT-PCR and IFA. An important concern in the diagnosis of ERhV1 based solely on the results of RT-PCR and IFA was that the virus detected may have represented persistent virus and therefore may not have been associated with the observed disease. Indeed, the ERhV1 genome was detected in nasopharyngeal swabs from two, now healthy horses from sample set 1 obtained 1 year after the occurrence of disease as well as from one of four nasopharyn-
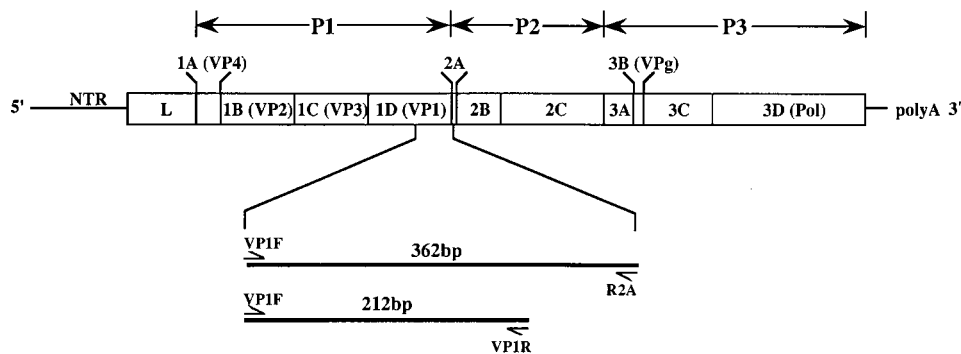

FIG. 2. Schematic diagram of the genome of ERhV1.393/76 (8). The locations of the primers used for RT-PCR and the expected sizes of the amplified products are shown at the bottom.
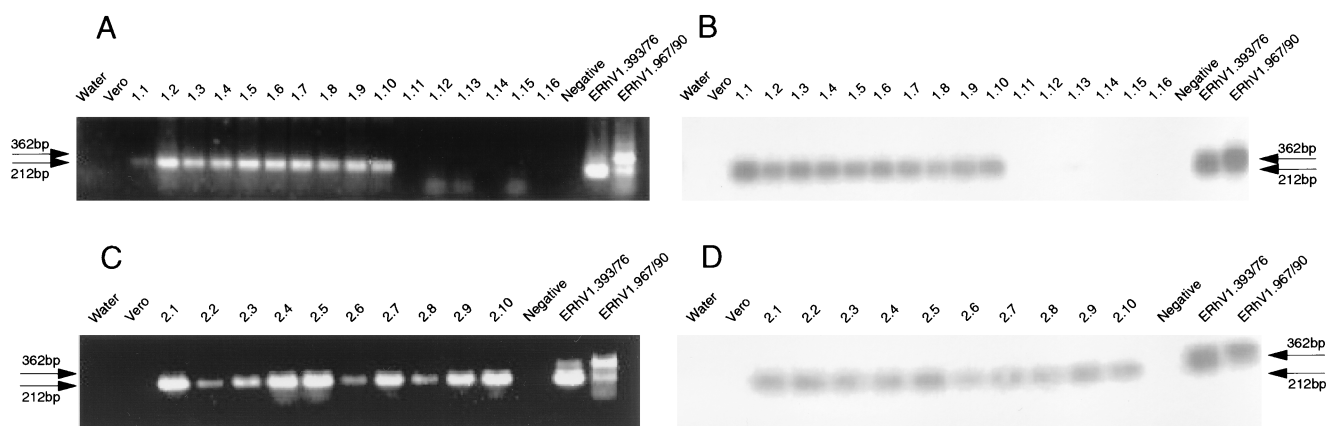
FIG. 3. Detection of ERhV1 RNA by RT-PCR in nasopharyngeal swabs collected from horses in two separate outbreaks (set 1, samples 1.1 to 1.16 [A and B]; set 2, samples 2.1 to 2.10 [C and D]) of respiratory disease. RT-PCR was performed and second-round PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide (A and C). Southern blot hybridization was performed on the second-round PCR products from A and C by using a VP1 probe (B and D, respectively). Also shown is RT-PCR amplification of RNA extracted from ERhV1.393/67-infected Vero monolayer cell cultures, ERhV1.967/90-infected Vero cells, uninfected Vero cells, a nasopharyngeal swab sample from an SPF foal infected with EHV2, and water only.

geal swabs from unrelated healthy horses on the same farm. However, in these instances only second-round PCR products were detected, and for this the more sensitive detection method of DNA hybridization was required. Detection of weak second-round PCR products in samples from some healthy horses probably represents the detection of low levels of persistent virus in the respiratory tracts of these horses. Alternatively, the possibility remains that these products represent a PCR artifact, although this seems unlikely because such products were not observed in any negative control samples.

First-round ERhV1 PCR products were detected in 12 of the 20 (60%) nasopharyngeal swabs from horses in sets 1 and 2 by ethidium bromide staining; first-round amplification of the 6 samples from apparently healthy horses was negative. This suggests that a positive first-round ERhV1 PCR product, detectable by ethidium bromide staining, in a nasopharyngeal swab from a horse showing symptoms of febrile respiratory disease may be used to tentatively diagnose ERhV1 infection. Confirmation of an ERhV1 diagnosis by IFA or retrospective serological examination of acute- and convalescent-phase sera for neutralizing antibody remains essential.

The detection of first-round PCR products in five of the retrospective clinical samples in set 3, which represented samples from horses involved in four different outbreaks, sug-

TABLE 2. SN antibody titers in sera obtained from mares (set 1)

Sample	Neutralizing antibody titer[a]			
	Day 0	Day 5	Day 20	Day 41
1.1	<200	800	2,300	6,300
1.2	<200	ND[b]	8,900	ND
1.3	ND	800	ND	3,200
1.4	ND	ND	3,200	8,900
1.5	ND	ND	800	4,500
1.6	ND	800	ND	4,500
ERhV1.393/76	800[c]			
NRS[d]	<200			

[a] Reciprocal of the highest dilution of serum that neutralized 100 50% tissue culture infective doses of ERhV1.393/76.
[b] ND, not determined.
[c] Neutralizing antibody titer of rabbit anti-ERhV1.393/76 serum.
[d] NRS, normal rabbit serum.

gested that ERhV1 was also the likely cause of respiratory disease in these horses and in their respective cohorts. Nasopharyngeal swabs taken from three horses gave first-round PCR products that were visualized by ethidium bromide staining; two of these (samples 3.3 and 3.6) were from horses involved in the same outbreak, strongly suggesting an ERhV1 etiology. ERhV1 was also detected after Southern hybridization of first-round PCR products in nasopharyngeal swabs collected from two other horses, indicating a possible ERhV1 etiology.

Each of the clinical samples described in this paper was inoculated onto EFK and/or Vero cells in an attempt to isolate virus. Primary isolation of ERhV1 from clinical specimens has proved most difficult in our experience. Since 1965 we have inoculated many filtrates of nasopharyngeal swabs from horses with acute febrile illness onto cell cultures but have isolated ERhV1, with considerable difficulty, on only two occasions, which gave rise to the strains ERhV1.393/76 and ERhV1.967/ 90. In these isolations, "abortive" infections with transient and variable degrees of CPEs were observed, especially in the first few days of incubation. The propagation of ERhV1.393/76 and ERhV1.967/90 was improved by switching from EFK to Vero cells, in which a complete CPE was observed. The rabbit kidney cell line RK-13 has been reported to be suitable for the isolation of some strains of ERhV1 (10). However, neither ERhV1.393/76 nor ERhV1.967/90 produced a CPE in RK-13 cells (data not shown). The reasons for the difficulties in isolating and propagating ERhV1 in cell culture remain unexplained and require further study.

The significant role of ERhV1 as a pathogen for horses reported in this study is in agreement with previous studies (1, 2, 20), and our results also suggest that disease caused by ERhV1 occurs far more frequently than has generally been considered. This, together with the recognition that ERhV1 is not a member of the *Rhinovirus* genus (viruses which cause relatively mild, strictly upper respiratory tract infection), but is most closely related to foot-and-mouth disease viruses, which, like ERhV1, produce a systemic viremic disease, has important implications. We also note and draw particular attention to the findings of Plummer (15), who showed that ERhV1 infects and causes disease in humans. In view of the distinct molecular and biological properties of ERhV1, further studies on the epide-

A

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            450       460       470       480       490       500
             •         •         •         •         •         •
ERhV1.393/76   TCCCGTTCACAAGCCTGTGCTTCAGCGTTGCCATATACATCAATGTGGCGTGTTGTGCCA
ERhV1.967/90   -----C--C-----G----GC-TCAATG----C--C--C--T-----------------
ERhV1.1090/94  -----G--C----------G--TCAACG------------C--------------C---
ERhV1.1165/95  ------------------GC-T---C--------------T------G--G------
               ***** ** ***** ****  *     **** ** ** ** ******** ** ** ***

            510       520       530       540       550       560
             •         •         •         •         •         •
ERhV1.393/76   GTCTTTTACAATGGCTGGGGTGCACCTACCAAAGAAAAGGCAACCTACAATTGGCTTCCT
ERhV1.967/90   --T--C-----C--T--------------A--------ATC--------------A--A
ERhV1.1090/94  --------------T--------T-----------G--------G-----------C---
ERhV1.1165/95  --C----------T-----A-------------------T-----------------G
               ** ** ***** ** ***** ** ***** ***** **  * ** ********** **

            570       580       590       600       610       620
             •         •         •         •         •         •
ERhV1.393/76   GGTGCACACTTTGGTTCCATCTTGCTGACTTCTGATGCGCATGATAAAGGAGGGTGCTAC
ERhV1.967/90   --------T-----C-----TC-------C--C-----C--C--C--G--C--T--T---
ERhV1.1090/94  --G--T---------------C----C-----C----------------T--T------
ERhV1.1165/95  -----------------------------------C-----------------T---
               ** ** ** ***** *****  **** ** ** ** ** ** ** ** ** ** ***

            630       640       650       660       670       680
             •         •         •         •         •         •
ERhV1.393/76   TTGCGGTATGCTTTCCGCGCGCCAGCGATGTATTGCCCTCGACCCATTCCGCCGGCTTTT
ERhV1.967/90   --CGC-GTAT-C-------GCG-C-------------------G--C-----T-----A
ERhV1.1090/94  ---------C-C-------GCG----C----------CA----A--------T--C--C
ERhV1.1165/95  --T-----C--A----CGCGA---AAC-------------C----------A--G--C
               **         * ****      *      ***********  * ** ** ***** ** **

            690       700       710       720       730       740
             •         •         •         •         •         •
ERhV1.393/76   ACGCGTCCAGCGGACAAAACCAGACATAAATTTCCCACTAACATCAACAAACAGTGTACT
ERhV1.967/90   --------G--------------G--C--G--C--T--C----------G-----C--C
ERhV1.1090/94  --TA-G--G--A--T-----G-----C--G--C-----G-----T-----G--------G
ERhV1.1165/95  -----G--T-----T--G--G--------G-----G-----T--T-----G---------
               **   *  *   ** ** ** ** ** ** ** ** ** ** ** ** ***** ***** **
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B

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            150       160       170       180       190       200
             •         •         •         •         •         •
ERhV1.393/76   SRSQACASALPYTSMWRVVPVFYNGWGAPTKEKATYNWLPGAHFGSILLTSDAHDKGGCY
ERhV1.967/90   ------GFNV---------------------------S---------------------
ERhV1.1090/94  ------GFNV------------------------------------------------
ERhV1.1165/95  ------GL--------------------------------------------------
               ******  .*************************.************************

            210       220       230       240
             •         •         •         •
ERhV1.393/76   LRYAFRAPAMYCPRPIPPAFTRPADKTRHKFPTNINKQCT
ERhV1.967/90   FAVS--GA-----------L--Q-----------------
ERhV1.1090/94  ---P--GA--------------------------------
ERhV1.1165/95  F----PR-N-------------------------------
                 *    ********** **  ****************
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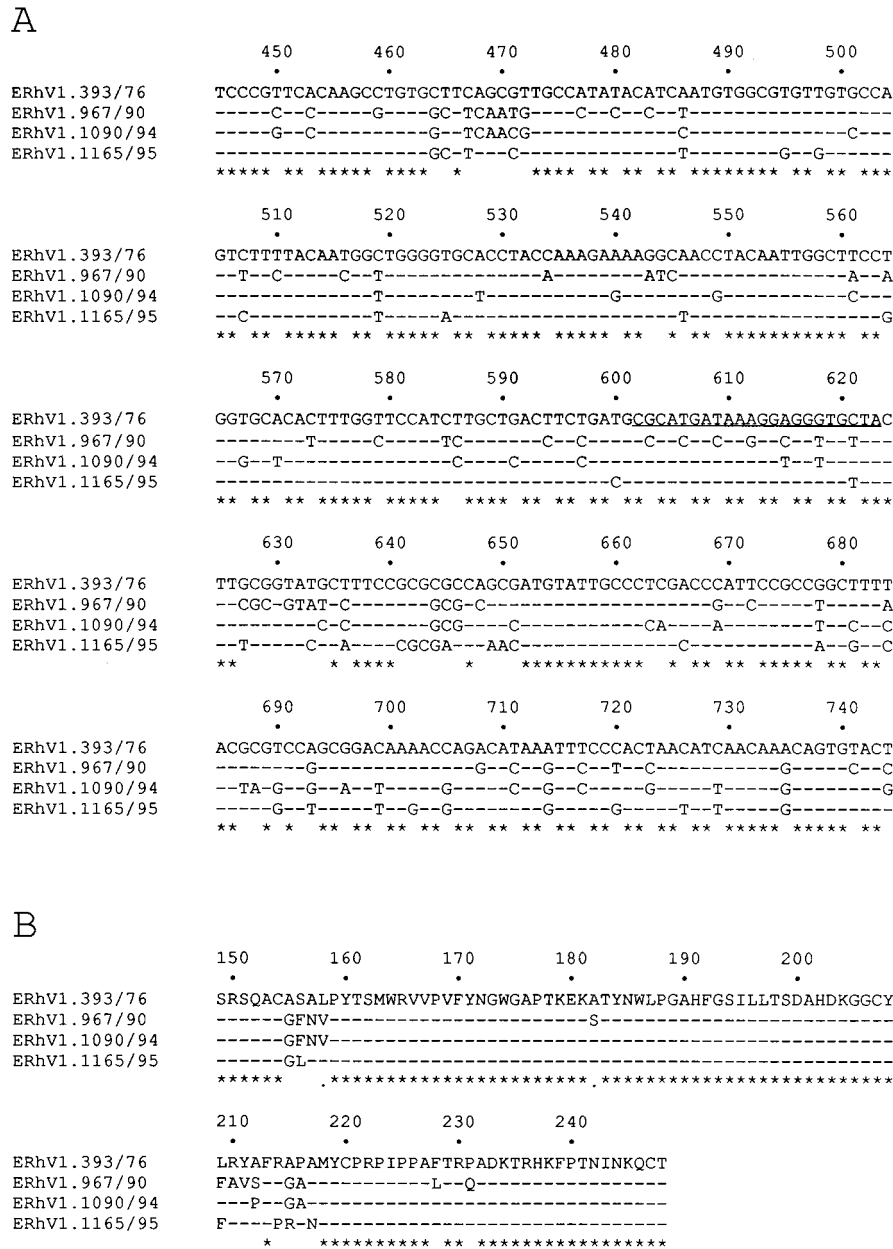
FIG. 4. Alignment of nucleotide sequences (A) and deduced amino acid sequences (B) of the 3′ regions of the VP1 sequences of ERhV1.967/90, ERhV1.1090/94, and ERhV1.1165/95 with the prototype strain ERhV1.393/76. The sequence of the internal PCR primer VP1R is underlined. Numbering of both nucleotides and amino acid residues is according to the published sequence for ERhV1.393/76 (8).

miology and disease associations of ERhV1 are needed to elucidate more clearly its role as a pathogen for horses and humans.

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