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# VIRULENCE AND VIREMIA CHARACTERISTICS OF 1992 EPIZOOTIC SUBTYPE IC VENEZUELAN EQUINE ENCEPHALITIS VIRUSES AND CLOSELY RELATED ENZOOTIC SUBTYPE ID STRAINS

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*Abstract.* Following a 19-year hiatus, Venezuelan equine encephalitis (VEE) reemerged in western Venezuela in December 1992. This outbreak is important in understanding VEE emergence because phylogenetic studies imply that sympatric, enzootic, subtype ID VEE viruses mutated to generate the epizootic/epidemic. Although the 1992–1993 strains belong to subtype IC, a serotype implicated in extensive outbreaks during the 1960s and in 1995, relatively small numbers of human and equine cases occurred in 1992–1993. We, therefore, evaluated the pathogenicity of these Venezuelan enzootic ID and epizootic IC viruses to determine 1) if they exhibit phenotypes like those described previously for more distantly related enzootic and epizootic strains, and 2) if the 1992–1993 outbreak was limited by the inability of these IC viruses to exploit equines as amplification hosts. All strains were virulent in mice and guinea pigs, but were benign for cotton rats, natural hosts of enzootic viruses. However, only the IC strains produced equine disease, with mean peak viremias of $10^5$ suckling mouse 50% lethal doses per mL serum, and some titers exceeding $10^7$. These viremias approximate those observed previously with VEE strains isolated during more extensive epizootics, suggesting that efficient equine amplification did not limit the scope and duration of the 1992–1993 outbreak. Enzootic ID virus infection protected all horses from challenge with epizootic strain P676, supporting the hypothesis that epizootics bypass regions of enzootic transmission due to natural immunization of equines by enzootic VEE viruses.

## INTRODUCTION

Venezuelan equine encephalitis (VEE) is an important emerging disease of humans and equines in many parts of the New World.[1–3] Sporadic outbreaks occurred during much of the twentieth century, affecting hundreds of thousands of people, horses, donkeys and mules, with high rates of mortality in equines and severe morbidity in humans. Venezuelan equine encephalitis outbreaks lasted up to several years and some spread over large geographic distances. For example, an outbreak that began in Guatemala and El Salvador in 1969 spread south to Nicaragua and north to Texas, and lasted three years.[2,4] The etiologic agent of these outbreaks, VEE virus, is a mosquito-borne virus in the Family Togaviridae, genus *Alphavirus*.[5,6] Venezuelan equine encephalitis virus strains implicated in most outbreaks belong to antigenic subtypes IAB and IC, while all other members of the VEE antigenic complex are considered enzootic viruses that circulate continuously, are generally avirulent for equines, and are incapable of epizootic amplification due to their inability to generate sufficient viremia in horses, donkeys, and mules.[1,7,8]

The discontinuous nature of VEE outbreaks resulted in many years of research to identify a source of the subtype IAB and IC viruses implicated in epidemics and epizootics. Although some outbreaks prior to 1980 may have been initiated by the use of incompletely inactivated vaccines made from virulent, epizootic IAB viruses,[9] the original evolutionary source of the IAB and IC viruses is believed to be enzootic, subtype ID viruses that circulate in northern South America.[10–12] The strongest genetic and epidemiological link between enzootic and epizootic VEE viruses is represented by subtype IC viruses implicated in a 1992–1993 epizootic and epidemic in western Venezuela,[13] and ID viruses isolated in the same region of Venezuela and in nearby eastern Colombia.[11] This outbreak followed 19 years of VEE inactivity, but involved relatively small numbers of confirmed human and equine cases compared to other epizootic/epidemics. Cases were first detected in December 1992 and the last confirmed infections occurred in July 1993. The etiologic subtype IC viruses share over 99% nucleotide and amino acid sequence identity with sympatric ID strains, and the closest enzootic relative identified to date has only 12 deduced amino acid differences in the complete structural and nonstructural polyprotein open reading frames.[12] This suggests that the 1992 VEE emergence resulted from the mutation of a local enzootic ID virus.

A possible explanation for the limited scope and duration of the 1992–1993 outbreak is that the subtype IC viruses responsible were not amplified efficiently by equines to allow for horse-mosquito-horse transmission, compared to genetically distinct IAB and IC viruses isolated during more extensive outbreaks. This hypothesis is also suggested by the greater genetic similarity of the 1992–1993 IC viruses to enzootic strains than is exhibited by any other epizootic viruses.[11,12] To test this hypothesis, we compared the pathogenicity of the 1992–1993 epizootic IC viruses with their sympatric ID relatives using rodents and equines. Results indicated that, although there is no consistent difference in rodent virulence, these viruses display typical equine virulence and viremia phenotypes following subcutaneous infection.

## MATERIALS AND METHODS

**Virus preparation, quantification, and serology.** The VEE virus strains used for experimental infections are described in Table 1. Baby hamster kidney (BHK-21) cells were infected at a multiplicity of approximately 0.1 to prepare virus stocks for experimental infections. Virus stocks

TABLE 1
Venzuelan equine encephalitis viruses used for experimental infections

| Strain | Subtype | Date of isolation | Place of isolation | Host | Passage history* |
|---|---|---|---|---|---|
| 66637 | ID | 19 November 1981 | Zulia State, Venezuela | Sentinel hamster | sm1, V1 |
| ZPC738 | ID | 24 September 1997 | Zulia State, Venezuela | Sentinel hamster | none |
| 243937 | IC | 18 December 1992 | Trujillo State, Venezuela | Horse | sm2 |
| SH3 | IC | 9 January 1993 | Trujillo State, Venezuela | Human | V1 |
| P676 (CBSI-9) | IC | August 1963† | Miranda State, Venezuela | *Anopheles triannulatus* mosquitoes | sm1, V1 |

* sm = suckling mouse; V = Vero cells.
† Exact date is not known.

and blood samples were titered by plaque assay on Vero cell monolayers overlaid with 0.4% agarose in Eagles minimal essential medium (MEM) supplemented with 2% fetal bovine serum (FBS), or by intracerebral inoculation of 1–3 day old suckling mice with serial 10-fold dilutions.

Seroconversion was detected using plaque reduction neutralization tests (PRNT) with the Trinidad donkey strain of VEE virus.[14] To ensure that previous alphavirus vaccination did not affect equine pathogenesis following VEE virus infection, pre-infection equine sera were screened for the presence of VEE, eastern (EEE) and western equine encephalitis (WEE) virus antibodies using three methods: 1) an ELISA with cell lysates prepared from BHK-21 cells infected with VEE virus strain Trinidad donkey,[14] EEE virus strain 82V2137,[15] and WEE virus strain Fleming;[16] 2) PRNT using the same virus strains; and 3) hemagglutination inhibition (HI) assays using suckling mouse brain antigens prepared from the same strains.

**Rodent infections.** Various strains and ages of mice (Table 2), guinea pigs, and cotton rats (Table 3) were infected by subcutaneous inoculation in the hind leg of 0.1 mL of MEM containing 5% FBS and 5–10 Vero cell plaque forming units (PFU) of virus. Blood samples were obtained from the saphenous vein at 24 hr intervals to quantify viremias and evaluate seroconversion.

**Horse infections.** Eight antibody-negative mares were identified that ranged in estimated age from 4 to 11 years. Only horses with no evidence of pre-existing alphavirus immunity were used for experimental infections. Serum taken 7–28 days after challenge was also tested using PRNT, HI, and complement fixation for the presence of EEE and WEE antibodies to identify anamnestic antibody responses in previously vaccinated animals whose antibody titers might have fallen below the detection limits of our pre-challenge assays. The mares were grouped in pairs, with each pair consisting of a younger and older mare, and the pairs were assigned at random to epizootic and enzootic virus strain infections.

All horses were infected by subcutaneous inoculation in the shoulder region of 1.0 mL of MEM containing 10% antibody-negative normal horse serum and 2,000 PFU of virus, a dose comparable to that inoculated by alphavirus-infected mosquitoes.[17] Although infection via the bite of an infected mosquito is known to potentiate viremia generated by some bunyaviruses,[18,19] we used needle inoculations for two reasons: 1) almost all past experimental equine infections with other epizootic VEE viruses have been conducted using needle infections, and we wanted to generate data that could be compared directly, and 2) biosafety considerations precluded the transport of infected mosquitoes into the large animal containment facilities.

Each pair of mares inoculated with a given virus was housed together in an isolation room within a large animal biocontainment building. Except for one animal that was not cooperative, rectal temperature was recorded twice daily up

TABLE 2
Viremia and survival of mice infected with enzootic subtype ID and epizootic subtype IC Venezuelan equine encephalitis viruses

| Mouse strain | Age (weeks) | Number tested | Virus strain | Virus subtype | Mean peak viremia ($Log_{10}$ Vero PFU/mL ± SD) | Mean survival time (days ± SD) |
|---|---|---|---|---|---|---|
| Balb/C | 4 | 4 | 66637 | ID | 5.5 ± 0.2 | 8.3 ± 0.5 |
| Balb/C | 8 | 3 | 66637 | ID | 4.6 ± 0.2 | 8.1 ± 1.0* |
| Balb/C | 4 | 4 | 243937 | IC | 5.5 ± 0.4 | 7.8 ± 1.5 |
| Balb/C | 8 | 4 | 243937 | IC | 5.2 ± 0.2 | 8.7 ± 0.4 |
| C57BL/6 | 4 | 4 | 66637 | ID | 5.8 ± 0.7 | 9.0 ± 0.0 |
| C57BL/6 | 8 | 3 | 66637 | ID | 5.7 ± 0.1 | 8.8 ± 0.4* |
| C57BL/6 | 4 | 4 | 243937 | IC | 5.4 ± 0.6 | 8.0 ± 2.3† |
| C57BL/6 | 8 | 4 | 243937 | IC | 5.6 ± 0.1 | 10 ± 0.0‡ |
| Swiss NIH | 4 | 4 | 66637 | ID | 6.4 ± 0.4 | 6.3 ± 0.5 |
| Swiss NIH | 8 | 3 | 66637 | ID | 4.7 ± 0.1 | 9.8 ± 1.4* |
| Swiss NIH | 4 | 4 | 243937 | IC | 6.9 ± 0.1 | 7.0 ± 1.2 |
| Swiss NIH | 8 | 4 | 243937 | IC | 5.8 ± 0.1 | 8.3 ± 1.0 |
| C3H | 4 | 4 | 66637 | ID | 5.6 ± 0.4 | 6.3 ± 0.5 |
| C3H | 8 | 4 | 66637 | ID | 4.8 ± 0.2 | 7.1 ± 0.2 |
| C3H | 4 | 4 | 243937 | IC | 5.5 ± 0.3 | 6.8 ± 0.5 |
| C3H | 8 | 4 | 243937 | IC | 4.9 ± 0.1 | 7.2 ± 0.2 |
| CD1 | 8 | 5 | 66637 | ID | 3.9 ± 0.7 | 5.0 ± 0.0 |
| CD1 | 8 | 4 | 243937 | IC | 4.9 ± 0.7 | 5.0 ± 0.0 |

* One mouse survived; † two mice survived; ‡ three mice survived. PFU = plaque forming units.

TABLE 3
Viremia and survival of Guinea pigs and cotton rats infected with enzootic subtype ID and epizootic subtype IC Venezuelan equine encephalitis viruses

| Animal | Strain | Age (weeks) | Number tested | Virus strain | Virus subtype | Mean peak viremia ($Log_{10}$ PFU/ml ± SD) | Mean survival time (days ± SD) |
|---|---|---|---|---|---|---|---|
| Guinea pig | Outbred | 10 | 2 | 66637 | ID | 5.6 ± 0.4 | 5.9 ± 3.3 |
| | Outbred | 10 | 2 | 243937 | IC | 5.8 ± 0.5 | 3.9 ± 2.5 |
| | Inbred 13 | 8 | 2 | 66637 | ID | 6.0 ± 0.4 | 7.3 ± 1.0 |
| | Inbred 13 | 8 | 2 | ZPC738 | ID | 6.6 ± 0.7 | 4.3 ± 0.4 |
| | Inbred 13 | 8 | 2 | 243937 | IC | 6.9 ± 0.1 | 4.6 ± 0.0 |
| Cotton rat | Virion | 4 | 4 | 66637 | ID | 5.2 ± 0.3 | >14 days |
| | Virion | 4 | 3 | 243937 | IC | 4.7 ± 0.2 | >14 days |

PFU = plaque forming units.

to 12 days beginning on the day prior to inoculation. Blood was collected twice daily during this same interval and serum was stored at −70°C for virus titration. Blood was also collected into EDTA-containing tubes every morning for up to 9 days beginning the day prior to inoculation, and analyzed using a QBC V hematology analyzer (Clay Adams). To determine whether natural enzootic infections protect horses from epizootic challenge, horses infected with subtype ID viruses were maintained for 28 days and then inoculated subcutaneously with 10,000 PFU of epizootic subtype IC strain P676, known to be equine-virulent.[7] Body temperature, serum collection, and hematological assays were performed following this second challenge as described above.

## RESULTS

**Rodent infections.** Peripheral challenge of all rodent species except for cotton rats generally produced death in under one week. Female mice of 4 or 8 weeks of age, both outbred and inbred strains, were tested. Both enzootic subtype ID strain 66637 and epizootic IC strain 243937 were equally lethal for outbred and inbred mice following inoculation with 5–10 PFU of viruses. Smaller doses resulted in inconsistent infection (data not shown). Signs of paralysis usually appeared on Day 5 post-infection, with outbred Swiss NIH and inbred CH3 strains being more susceptible to this disease manifestation. The average survival time of Swiss NIH and inbred CH3 mice was also shorter than Balb/C and C57BL/6 strains (Table 2). Mouse viremia of $10^4$ to $10^5$ PFU/mL of blood was generally detected one day after infection, and remained high through Day 4. All of these results are consistent with those of previous murine studies using epizootic VEE virus strains.[20–23] Guinea pigs also had uniformly fatal infections (Table 3); although they displayed some variability in survival time and viremia, there was again no consistent difference in response to the enzootic versus epizootic VEE virus strains. In contrast to mice and guinea pigs, female cotton rats had relatively high viremia titers, but did not show any clinical signs of infection, and all survived for 14 days (Table 3).

**Horse infections.** Eight horses were identified as antibody-negative based on the following criteria: 1) PRNT titers of < 1:20 for VEE, EEE, and WEE viruses; 2) ELISA reactions yielding absorbence values within 2 standard deviations of the mean for negative control horse serum; and 3) HI titers < 1:20. Following infection, all horses that survived developed neutralizing antibodies > 1:20 against VEE virus, but only one (infected with ID strain ZPC738) showed any sign of a rise in the EEE or WEE virus antibody titer (an HI titer of 1:80 to EEE virus, compared to titers of 1:20–1:40 for control VEE-specific mouse antisera). This horse may have previously been immunized against EEE and/or WEE virus, or may simply have produced more cross-reacting antibodies than the other horses, following VEE virus infection.

Two horses were infected with each of 2 enzootic subtype ID strains (66637 and ZPC738) and two each with subtype IC epizootic strains (SH3 and 243937). Table 4 summarizes the signs observed in the horses. None of the horses infected with the enzootic, subtype ID strains exhibited any overt

TABLE 4
Signs in horses infected with Venezuelan equine encephalitis viruses

| Sign/symptom | Fraction displaying sign | |
|---|---|---|
| | Enzootic subtype ID | Epizootic subtype IC |
| Depression | 0/4 | 4/4 |
| Reluctance to move | 0/4 | 4/4 |
| Unresponsiveness | 0/4 | 1/4 |
| Ataxia | 0/4 | 1/4 |
| Anorexia | 0/4 | 4/4 |

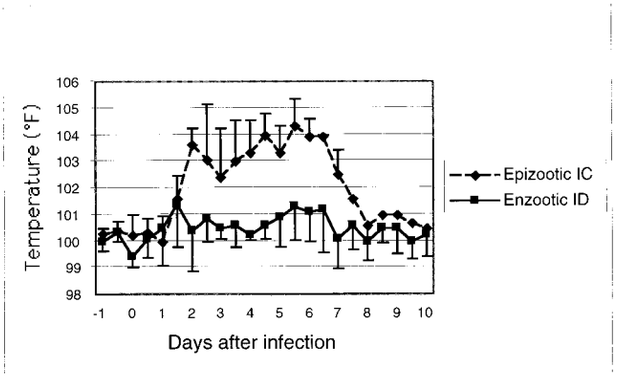


FIGURE 1. Febrile responses of equines infected with enzootic subtype ID and epizootic subtype IC Venezuelan equine encephalitis viruses. Data represent means of four mares infected with two different enzootic strains (66637 and ZPC738) and three mares infected with two different epizootic strains (SH3 and 243937). Bars indicate standard deviations.

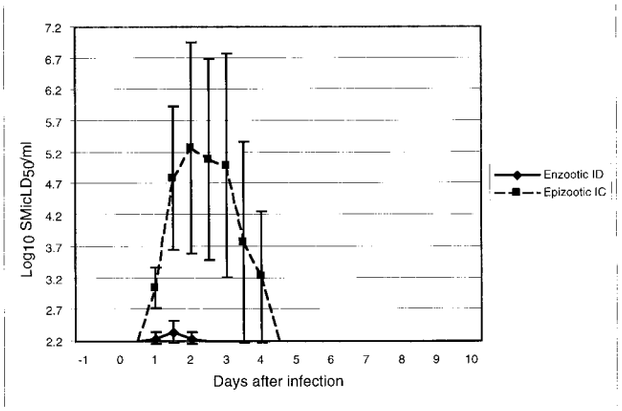


FIGURE 2. Viremia in equines infected with enzootic subtype ID and epizootic subtype IC Venezuelan equine encephalitis viruses. Data represent means of four mares infected with two different enzootic strains (66637 and ZPC738) and two different epizootic strains (SH3 and 243937). A $\log_{10}$ titer of 2.2 suckling mouse $LD_{50}$ represents the maximum sensitivity of the assay. Bars indicate standard deviations.

signs associated with VEE, and their body temperatures remained stable during the week following virus inoculation (Figure 1). In contrast, all 4 horses infected with the subtype IC strains exhibited one or more signs previously associated with equine VEE disease, and each of the three animals from which temperatures were recorded developed a febrile response (Figure 1). Due to the severity of clinical signs, one mare inoculated with IC strain SH3 was killed on Day 5 and one inoculated with IC strain 243937 was killed on Day 7. The other two mares inoculated with IC viruses were killed on Day 10, by which time they were clinically normal.

Hematological abnormalities were observed in all mares between Days 2 and 7 after virus inoculation (inoculation was on Day 0). Specifically, they showed suppressed hematocrit, leukopenia, and thrombocytopenia ($P < 0.05$; SAS repeated measures ANOVA), and despite the marked difference in clinical signs, there were no differences in these parameters between animals inoculated with epizootic versus enzootic viruses.

Only one horse infected with an enzootic subtype ID strain (66637) developed viremia detectable in our Vero cell plaque assay, at 4 PFU/mL after 48 hr of infection (data not shown). When sera were assayed using intracerebral inoculation of 1–2 day old mice, three of four enzootic infections had detectable viremia, with a mean peak titer of 2.4 $Log_{10}$ $SMicLD_{50}$/mL, 36 hr after infection (Figure 2). In contrast, all four horses infected with epizootic strains had detectable viremia lasting from 60–84 hr. Peak titers were 1.8 $Log_{10}$ PFU/mL and 5.3 $Log_{10}$ $SMICLD_{50}$/mL 48 hr after infection, and viremia was not detected more than 96 hr after infection (Figure 2).

All horses were tested for seroconversion using PRNT. The animals infected with IC viruses were tested coincidentally with euthanasia, which was 5 or 7 days post-inoculation for the 2 moribund cases, or 10 days post-inoculation for the two horses that recovered from acute disease. Horses infected initially with subtype ID viruses were tested immediately before challenge with strain P676. All eight horses seroconverted as indicated by the presence of neutralizing antibody titers of 1:40 or greater.

All four horses infected with subtype ID viruses were challenged with $10^4$ PFU of epizootic subtype IC strain P676. None developed any clinical signs of disease, hematological abnormalities, or detectable viremia, and all were killed 7 to 9 days after challenge.

## DISCUSSION

Previously described experimental infections of equines with epizootic subtype IAB and IC VEE viruses generally resulted in high rates of overt disease with high titered viremias, often reaching $10^{5–8}$ $SMicLD_{50}$/mL serum.[2,7,8,24–28] Equine mortality rates in these previous studies were generally approximately 50%, similar to those measured during epizootics. In contrast, enzootic VEE viruses belonging to subtypes ID,[7] IE,[29] II,[24] III, and IV[30] generally produce little or no clinical illness and viremia less than $10^5$ $SMicLD_{50}$/mL serum following experimental equine infection. These enzootic viruses also have not generally been associated with equine disease in nature. Our results with subtype IC strains isolated during a relatively small outbreak in western Venezuela, and closely related, sympatric enzootic subtype ID isolates, are consistent with these previous findings. Our equine viremia data do not support the hypothesis that the duration or scope of the 1992–1993 outbreak was related to lack of efficient equine amplification.

Other possible explanations for the limited nature of the 1992–1993 outbreak include inadequate populations of competent vectors and limited populations of susceptible equines. The recent completion of a dam and reservoir near the 1992 Trujillo epicenter suggests the possibility that alterations in the local mosquito fauna or populations may have facilitated epizootic transmission. Unfortunately, vector surveillance was not conducted in the affected regions of Venezuela before or during the epidemic, and it is, therefore, not possible to estimate if vector populations may have been a limiting factor during the outbreak. Equine vaccination coverage was probably very poor in 1992 because VEE activity had not occurred in Venezuela for 19 years.[1,3] Although vaccination efforts have failed to limit some past outbreaks to small geographic areas like those affected by the 1992–1993 epidemic, it is always difficult to assess the role of vaccination in limiting VEE outbreaks.[1]

Most of the major VEE epizootics in Venezuela have involved the Guajira Peninsula, the home of a large feral donkey population that is impossible to vaccinate effectively, while the Trujillo region is inhabited only by domestic equines residing on farms. This may have resulted in more complete vaccination coverage once the 1992–1993 outbreak was recognized. The failure of this outbreak to spread to the Guajira region may have been another limitation on the scope of the epizootic.

Another factor that may have limited the 1992–1993 outbreak is natural immunity in equine populations living close to enzootic foci in southwestern Venezuela. There is a lack of historical evidence for VEE epizootics in the Catatumbo and Sur del Lago regions of Zulia State, just west of the 1992–1993 epicenter. This region includes many foci where enzootic subtype ID viruses circulate.[12,39–41] Our demonstra-

tion that these ID viruses fully protect horses against challenge with epizootic strain P676, known to be virulent for naïve horses,[7] supports the hypothesis that epizootics rarely involve regions of enzootic transmission because of natural enzootic equine immunization. Renewed vaccination following the 1992–1993 and 1995 outbreaks precludes the evaluation of natural equine immunity in the Trujillo region at the present time.

Because many mosquito vectors implicated in epizootics are relatively insusceptible to systemic infection leading to transmission,[31,32] high levels of equine viremia are believed to be required for efficient amplification during outbreaks. This limited vector susceptibility, combined with little or no equine viremia following enzootic infections, explains the inability of enzootic VEE virus strains to generate epidemics and epizootics like serotypes IAB and IC.[1–3] Our results of little or no viremia in equines following infection with ID viruses supports this assumption. Humans also develop relatively high titered viremia following epizootic virus infection[33–35] but are probably less important for amplification because they are less attractive and subject to far fewer mosquito bites than equines. Although human infections with enzootic VEE viruses can produce severe and sometimes fatal disease,[36–38] equines living near enzootic foci develop VEE-specific antibodies but apparently do not often suffer disease.

The close genetic and epidemiological relationship between subtype IC strains isolated during the 1992–1993 Venezuelan outbreak and sympatric, enzootic ID isolates provides a unique model system for identifying mutations that can produce equine-virulent, epizootic viruses from enzootic progenitors. These mutations are hypothesized to have occurred in 1992 in western Venezuela.[11–13] An infectious cDNA clone generated from ID strain 66637 is now being used to test putative epizootic mutations predicted by genomic sequences and phylogenetic methods.[12] Unfortunately, rodent models used previously to assess VEE virus virulence and epizootic potential[21,23,42–44] do not appear to be useful in predicting equine avirulence of the enzootic Venezuelan subtype ID strains that we tested. Guinea pigs have been shown to respond differently to some enzootic VEE virus strains than to epizootic serotypes, but this pattern does not always extend to the ID subtype.[44] Our results support this previous conclusion. Mice, which have been useful models for laboratory attenuation studies,[21–23] do not appear to be predictors of natural equine virulence. Although several *in vitro* markers including plaque size on Vero cells overlaid with unpurified agar,[12,25] hydroxylapatite chromatography elution profiles,[45] and interferon $\alpha/\beta$ sensitivity[46,47] are useful in predicting the epizootic phenotype, experimental infections of equines will continue to be required in the definitive assessment of epizootic potential.

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