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Polymerase Chain Reaction Species Diagnostic Assay for Anopheles quadrimaculatus Cryptic Species (Diptera: Culicidae) Based on Ribosomal DNA ITS2 Sequences

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ABSTRACT Species-specific differences in the nucleotide sequences of the 2nd internal transcribed spacer (ITS2) of nuclear ribosomal DNA (rDNA) were used to develop a diagnostic assay based on the polymerase chain reaction (PCR) that can distinguish 4 of the 5 cryptic sibling species in the common malaria mosquito, Anopheles quadrimaculatus Say, complex. The assay requires only a small amount of tissue from an individual mosquito and a mixture of 5 PCR primers. The plus strand universal primer is derived from a sequence in the 5.8S coding region that is identical in all members of the complex. The 4 minus strand primers were selected from species-unique sequences within the ITS2 region. PCR amplification produces a different sized fragment for each of the 4 species which can be visualized readily under ultraviolet light after electrophoresis through an ethidium bromide-containing agarose gel. The assay has been developed and tested only with An. quadrimaculatus complex specimens from Florida populations.

KEY WORDS Anopheles quadrimaculatus, polymerase chain reaction, ribosomal DNA, cryptic species

The common malaria mosquito, Anopheles quadrimaculatus Say, once the most important vector of malaria in the eastern United States, was described in 1824 by Say (Knight and Stone 1977) and occurs throughout the eastern United States extending westward to Texas and the Dakotas. Recently, evidence from population crossing experiments, cytogenetics, and allozyme studies has revealed that this taxon is a complex of 5 cryptic species, currently called species A, B, C1, C2, and D (Kaiser et al. 1988a-c; Lanzaro et al. 1988; Narang et al. 1989a, b, 1993).

Before eradication of malaria from the United States around the middle of this century, An. quadrimaculatus was recognized as the major vector of human malaria parasites in the eastern United States (Boyd 1949). Because species A is the most widespread member of the An. quadrimaculatus complex (Johnson et al. 1993), it may have been responsible for most of the transmission of malaria. Although malaria has been eradicated, An. quadrimaculatus sensu lato still occurs in high numbers in some areas and is an important seasonal pest species (Kaiser et al. 1988a,b). In addition, the risk of local transmission of introduced malaria is increasing in many parts of United States, particularly in southern states which are visited by increasing numbers of malaria-infected migrant workers from Central and South America (Maldonado et al. 1990, Brook et al. 1994). In the event of such local outbreaks, incrimination of the vector and subsequent control will depend on techniques that can be used for rapidly identifying different member species in the An. quadrimaculatus species complex.

No obvious morphological differences exist that can be used to distinguish individual specimens of the 5 species in the An. quadrimaculatus complex; however, a number of biochemical, cytogenetic, and molecular diagnostic techniques have been developed. Species A and B can be distinguished by the presence of fixed inversions on the polytene chromosomes found in ovarian nurse cells (Kaiser et al. 1988b). The ovarian nurse cell polytene chromosomes of species C1, C2, and D are absent or have diffuse bands that cannot be resolved predictably (Kaiser et al. 1988b, Narang et al. 1989b). Larval salivary gland polytene chromosomes can be used for distinguishing these latter 3 species (P. E. Kaiser, personal communication).

All species in this complex can be identified by means of diagnostic electrophoretic allozymes (Narang et al. 1989a-c, 1990). An allozyme-based dichotomous key for distinguishing species A, B, C (C1 or C2), and D has been developed that uses 4 enzyme systems representing 5 genetic loci. An ad-
Diagnostic PCR reactions were conducted on specimens from the following localities in Florida: species A, Lake Rousseau; species B, Lake Octahatchee; species C1, Bear Bay Swamp, and species C2 and D, Choctawhatchee River. This material was F1 progeny reared from field-collected females that were chromosomally and electrophoretically identified by the staff at USDA in Gainesville, FL.

**Determination of ITS2 Sequences.** Procedures for DNA extraction, PCR amplification, purification, and DNA sequencing were as described by Porter and Collins (1991). The ITS2 region and flanking sequence were amplified by PCR using the primers previously described by Porter and Collins (1991) and Wesson et al. (1992). The plus strand primer was complementary to 20 nucleotides of the 3’ end of the 5.8S rDNA and contained an additional set of 8 nucleotides that included the recognition sequence for the restriction enzyme KpnI. The minus strand primer was complementary to 20 bases of the 5’ end of the 28S rDNA region and had 8 nucleotides containing the recognition sequence for the restriction enzyme KpnI. At least 2 ITS2 clones were sequenced for each species. In total, 12 clones were sequenced: 1 clone from each of 3 different individuals of species A, 3 clones from a single specimen of species B, 2 clones from single specimens of each of the species C1 and D, and 1 clone from each of 2 specimens of species C2. Sequences were aligned with the aid of the multiple alignment program Pileup (GCG Sequence Analysis Software Package, Genetics Computer Group, Madison, WI). Final alignment was produced visually following the general rule that single gaps were subdivided only when each additional gap facilitated alignment of a block of 3 or more consecutive nucleotides (Fig. 1).

**Selection of Species-Diagnostic Primers.** Differences in the ITS2 sequences of C1 and C2 were not sufficient to allow these species to be distinguished in the PCR diagnostic assay described here. The criteria considered for the selection of the diagnostic primers were (1) each species-specific primer differed from the homologous sequence in the heterologous species by 2 or more nucleotides at the 3’ end of the primer, (2) all primers had similar melting temperatures \( T_m \) = the midpoint in the temperature range over which melting of the primer from its complementary DNA strand occurs, (3) all primers had low secondary structure folding tendencies, and (4) primers would amplify species-specific fragments sufficiently different in size to be resolved on a 2% agarose gel. The \( T_m \)s of the primers were calculated by the computer program Oligo 3.3 (Rychlik and Rhoads 1989).

**Polymerase Chain Reaction Method.** Entire genomic DNA extractions from mosquitoes fol-

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**Materials and Methods**

**Material Examined.** All specimens of the *An. quadrimaculatus* complex were from Florida and were provided by the United States Department of Agriculture, Agricultural Research Service Medical and Veterinary Entomology Research Laboratory (Gainesville, FL). The specimens used for sequencing were from the following localities: species A, laboratory colony of the Orlando strain, Gainesville, FL; species B, Lake Octahatchee, Florida; species C1, Bear Bay Swamp, Florida; and species C2 and D, Choctawhatchee River, Florida.

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**Fig. 1.** Alignment of the \( f \) of rDNA from the 5 species. Bold letter designation of the spe and 5’ nucleotide of the 28S in these positions. These parts o for the PCR assay. These are U32530.
PCR of A. quadrimaculatus Cryptic Species

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15 were conducted on five localities in Florida: species A, Lake Ohrid, species B, Lake Octa­
Bay Swamp, and species C, Lake Rambler. This material was field-collected females and electrophoretically
analyzed at USDA in Gainesville, FL.

sequences. Procedures for PCR amplification, puri­
fication were as described by Porter and

et al. (1992). The plus 5.8S rDNA and contained
the restriction enzyme KpnI.

sequences were sequenced for each
were sequenced: 1 single specimen of species B
clone from each of 2 spec­

e was used to allow these species to be
derived from the homologous
ous species by 2 or more

3) all primers had low sec­
gs were subdivided only
pip facilitated alignment of
secutive nucleotides (Fig. 1).

Diagnostic Primers. Diff­
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sequences of C1 and
allow to specify these species to be
diagnostic assay derived
considered for the selection
of the homologous
ous species by 2 or more

end of the primer, (2) all
m = temperature range over which
from its complementary
(3) all primers had low sec­
gs, and (4) primers spe­
F to be resolved on a 2%
10
g of the primers were calculated

Reaction Method. Entire
ions from mosquitoes fol­
lowed the method described in Collins et al. (1987). The PCR reagents, including the deoxy-nucleotides, 10× buffer, polymerase, and the MgCl₂, were all obtained from the Perkin-Elmer Cetus GeneAmp PCR Reagent kit with AmpliTaq DNA polymerase (Norwalk, CT). The amount of these reagents was based on that found to be optimal for the PCR assays (per 25 μl of reaction mixture) for identification of individual mosquitoes of the An. gambiae complex (Scott et al. 1993). However, several reactions were conducted to determine amount of template DNA and relative concentration of primers that would differentiate both species and potential hybrids within the An. quadrimaculatus complex. Hybrid samples were simulated by using equal amounts of extracted genomic DNA from 2 species as the template. Each sample was amplified for 25 cycles in a Perkin-Elmer Cetus thermal cycler providing denaturation at 94°C for 1 min, annealing at 50°C for 2 min and extension at 72°C for 2 min. On completion, 15 μl of the reaction mixture plus 6 μl loading dye (0.25% bromophenol blue, 0.25% xylene cyanole FF, and 30% glycerol in water; Sambrook et al. 1989) were electrophoresed through a 2% agarose gel containing ethidium bromide. PCR fragments were resolved by ultraviolet light illumination. The initial relative concentrations of the 5 diagnostic primers were calculated on the basis of their Tₘs, assuming that the PCR would generally require higher concentrations of those primers with lower Tₘs. Optimal primer concentrations were determined empirically using the criteria that PCR reactions with template DNA from 2 different species (a simulated hybrid) would produce the 2 species-specific bands in approximately equimolar amounts. Once the optimal reaction mixture was obtained, the consistency and specificity of the assay was tested on both extracted DNA at different concentrations and on mosquito legs. A leg was triturated with 1 μl of sterile water in the bottom of the PCR reaction tubes and this triturate was used as template DNA.

Results

Alignment of ITS2 Region. Fig. 1 shows the alignment of the ITS2 and flanking 5.8S and 28S regions of the 5 members of the An. quadrimaculatus complex. The alignment in the 1st ⅔ of the ITS2 region was more or less unambiguous but became more equivocal in the latter ⅓ due to substantial divergence among the species in this region.

Fig. 2. Predicted folding structure of the 3’ end of 5.8S and 5’ end of 28S regions of An. quadrimaculatus species A. Numbering corresponds with that in Fig 1.

The approximate boundaries of the ITS2 region are defined by base pairing between the 3’ end of the 5.8S and the 5’ end of the 28S genes (Fig. 2). Fig. 2 also shows the loop which in many Diptera other than mosquitoes is cleaved to form the break between the 5.8S and 28S regions (Pavlikas et al. 1979, Porter and Collins 1991, Wesson et al. 1992).

No ITS2 clonal variation was found in species A, C₁, C₂, and D. One clone sequenced from species B differed from the other 2 by a single transition, where a thymine was replaced by a cytosine at position 331. The 2 clones of species D differed by 1 transition in the loop that results in the 2S break in higher Diptera like Drosophila melanogaster L. (Pavlikas et al 1979). At position 62, one clone had an adenine and the other a guanine.

The nucleotide length of the ITS2 region, demarcated by the asterisks in Fig. 1, ranged from 287 (species A), 298 (species D), 306 (species C₂), 318 (species C₁) to 329 (species B). Length variability among the 5 species was caused predominately by insertions and deletions (indels), particularly in the region that included and immediately flanked the CAG repeat motif near the 3’ end of the ITS2 sequence (Fig. 1). Species C₁ and C₂ differed from each other only in the number of CAG repeats. Of the 366 positions (including indels, each position of which is counted) required to align all the ITS2 sequences of the 5 An. quadrimaculatus cryptic species, 37.7% (138) were variable. Indels in ≥1 species were present at 29.2% (107) of the sites, and 8.5% (31) of the sites without indels differed from the consensus in ≥1 of the species. These differences included 15 transitions and 16 transversions, and all 4 of the differences that directly flanked an indel were transversions.

The variable regions were not distributed uniformly throughout the ITS2 sequences but were concentrated in 2 types of regions. Most of the variation was in the region that included and flanked the CAG repeats. In the secondary folding structure assumed by the ITS2 sequences of these species (not shown), this region is the central hub where most of the major stems meet, and many of the species-specific differences are present in unpaired regions. The large difference among species is due to rearrangements in folding. Most of the other areas of those involving indels, correspond to secondary structure motifs.

A large number of tandem repeats are dispersed throughout the ITS2 region. These repeats may underlie degree of sequence divergence. The dinucleotides CCG and AG are abundant. The CAG motif is present in different locations within the An. quadrimaculatus B and C₁ species. The most obvious tandem repeat is the CAG repeat region. At position of times this motif was represented by species in both species. Some of the indels flank the CAG repeats, not the degeneration of the repeat motif that compensate for slipped slippage, and some, for example, is possible that the dinucleotide positions 175-176 results of slippage at CG to AG, with some large indels 5’ of the CAG motif affect slippage and subsequent short tandem motifs nucleotides whereas the indel 3’ to have been caused by slipped motif.

Selection of Primers. An. quadrimaculatus species and species-specific primers were designed for underlined areas in (Fig. 1). Nucleotides in length; Tₘ = 59.8°C (Table 1). The size of amplified fragments were 26 bp for species B, 293 bp and 141 bp for species D.

Polymerase Chain Reaction. Recommended concentrations 25 μl species-diagnostic primer = 1–5 ng mosquito DNA (DNA extracted from a 0.10× fraction buffer nanopearl.
An. quadrimaculatus species

Table 1. Primers used for PCR amplification of fragments of the ITS2 region for species-diagnostic assay

<table>
<thead>
<tr>
<th>Species</th>
<th>Oligonucleotide sequence (5' to 3')</th>
<th>Length of fragment amplified</th>
<th>Tm °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR primers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UNAQ</td>
<td>CGA CAC AGC TCG ATG TAG AC</td>
<td>51</td>
<td></td>
</tr>
<tr>
<td>AQA</td>
<td>TCG GTA CGA GCC TGC ATT TT</td>
<td>319</td>
<td>56</td>
</tr>
<tr>
<td>AQB</td>
<td>CAC ACT ACA CAA CAG GCT TT</td>
<td>227</td>
<td>47.4</td>
</tr>
<tr>
<td>AQC</td>
<td>TAC CCC GGC CTT GTA GCA AA</td>
<td>263</td>
<td>55.9</td>
</tr>
<tr>
<td>AQD</td>
<td>ATG CAA AAG GTG GTG TGG TG</td>
<td>141</td>
<td>52.9</td>
</tr>
</tbody>
</table>

Included are the expected species-specific fragment sizes and Tm of the diagnostic primers. UNAQ, universal primer; AQA, An. quadrimaculatus species A primer; AQB, An. quadrimaculatus species B primer; AQC, An. quadrimaculatus species C and C4 primer and AQD, An. quadrimaculatus species D primer.

A large number of tandem repeat motifs were dispersed throughout the ITS2 sequences, and these repeats may underlie the relatively large degree of sequence divergence between these species. The dinucleotides GT and GC were very abundant. The CAC motif was repeated in 3 different locations within the ITS2 region of An. quadrimaculatus B and twice in the other species. The most obvious tandem array of repeats consisted of the CAG repeat region where the number of times this motif was repeated varied in the 5 species. Some of the indels, in particular those that flank the CAG repeats, may have resulted from degeneration of the repeat sequence as mutations that compensate for slippage accumulate. For example, it is possible that the indels occurring from nucleotide positions 175–182 (species D) were as a result of slippage at CG and CAC repeats. The large indels 5' of the CAG repeat region may reflect slippage and subsequent degeneration of short tandem motifs such as CAC and CGT, whereas the indel 3' to the CAG repeat region may have been caused by slippage of a TACA repeat motif.

Selection of Primers. Locations of the universal and species-specific primers are shown as the underlined areas in (Fig. 1). Each primer was 20 nucleotides in length; Tm ranged from 47.4 to 58.9°C (Table 1). The sizes of the species-specific amplified fragments were 319 bp for species A, 227 bp for species B, 253 bp for species C and C4, and 141 bp for species D (Figs. 1 and 3).

Polymerase Chain Reaction Conditions. Recommended concentrations of PCR reagents for a 25 μl species-diagnostic PCR assay are as follows: 1–5 ng mosquito DNA (1/1,000 part of the total DNA extracted from a single mosquito), 2.5 μl 10× reaction buffer number II, 200 μM each dNTP, 2 mM MgCl2, 0.625 units AmpliTaq polymerase and sufficient sterile water to make a final reaction volume of 25 μl (all components except DNA from Perkin Elmer Cetus). The following primer concentrations consistently produced visible bands that distinguished both hybrids and non-hybrids: 1.6 pmol (10 ng) of AQU, 1.0 pmol (6 ng) of AQA, 4.6 pmol (28 ng) of AQB, 2.3 pmol (14 ng) of AQC, and 5.4 pmol (34 ng) of AQD (Fig. 3).

A 1/1,000 dilution of DNA extracted from an individual mosquito (estimated at 1–5 ng) produced PCR products that were easy to resolve in an ultraviolet light illuminated, ethidium bromide stained agarose gel. Increasing the amount of DNA by 10-fold resulted in no improvement in band intensity. All simulated hybrid combinations (except those involving C2 which were not tested) produced the 2 species-diagnostic bands in roughly equal intensity (Fig. 3, lanes 6–11).

Discussion

Sequences of ITS2. Lengths of the ITS2 region in the members of the An. quadrimaculatus complex (287 bp in species A to 329 bp in species B) are not unlike those of other North American members of the Anopheles maculipennis Meigen complex such as Anopheles hermsi Barr & Gutiavajin (305), An. freebomi (310) and Anopheles occidentalis Dyar & Knab (306) (Porter and Collins 1991). These lengths are similar to those of the ITS2 sequences of other Anopheles, Aedes, Haemagogus, and Psorophora mosquito investigated thus far, which fall in the approximate length range of 300–500 bp (Wesson et al. 1992, Paskewitz et al. 1993, Fritz et al. 1994).

The GC content of the An. quadrimaculatus complex ITS2 sequences ranged between 55.1% for species D and 57.3% for species B. These per-
centages are very similar to the ITS2 GC contents of other anopheline and culicine mosquito species, which all fall in the range of 50-60% (Porter and Collins 1991, Wesson et al. 1992, Paskewitz et al. 1993, Fritz et al. 1994), but they are markedly higher than the GC content of the ITS2 sequences of the flies D. melanogaster and Sciara coprophila, which range between 20 and 30% (Jordan et al. 1980, Tautz et al. 1988). Schlötterer et al. (1994) proposed that the high A-T content of Drosophila ITS2, which is unusual for noncoding DNA, was because the rDNA genes were located in the centromeric heterochromatin. However, rDNA genes of all Anopheles studied to date, including An. quadrimaculatus A and members of the An. gambiae complex, are located in the heterochromatin associated with the X or Y and X chromosomes (Collins et al. 1989, Kumar and Rai 1990).

Pairwise sequence differences among all the species in the An. quadrimaculatus (total number of nucleotide positions that are either different or opposite a gap divided by the total number of bases required to align the 2 sequences) are unusually high for cryptic species, ranging from 18.5% between species A and B to 25.7% between species B and D. These differences are almost an order of magnitude higher than known measures of ITS2 sequence divergence within other cryptic Anopheles complexes. For example, in a comparison of ITS2 sequences of 5 species in the An. gambiae cryptic species complex, differences ranged from a low of 0.4% to a high of 1.6% (Paskewitz et al. 1993). The cryptic sibling species An. hermsi and An. freeborni differed by only 4.9% in their ITS2 sequences, and the more distantly related and morphologically distinguishable An. occidentalis differed from An. hermsi at only 11.5% of the ITS2 positions (Porter and Collins 1991).

The large amount of ITS2 interspecies variation in the An. quadrimaculatus complex is not explained simply by evolutionary divergence. For example, in sequence comparisons involving part of the 28S rRNA expansion domain 2 coding region, species A and B of the An. quadrimaculatus complex differed at only 1 position, a level of divergence that was essentially the same as that observed in comparisons of An. freeborni and An. hermsi (1 position) or among some members of the An. gambiae complex, which differed at a maximum of 2 positions (C.H.P. and F.H.C., unpublished data).

We suspect that this high level of ITS2 sequence divergence observed in the An. quadrimaculatus complex may be caused, in part, by their simple subrepeat structure and the propensity of such sequences to experience replication slippage. The phenomenon has been well documented, even on the intraspecific level. For example, in their study of ITS2 sequences from different populations of Anopheles nuneztovari Gabaldon, Fritz et al. (1994) observed indel differences in 3 different regions, and 2 of these regions were GA dinucleotide repeat tracts. The pattern observed in the An. quadrimaculatus complex may be typical of the kind of saltatory sequence divergence that can accompany expansion or contraction of short nucleotide repeat domains. The most pronounced interspecies sequence differences actually occur in the CAG repeat domains and flanking sequences (Fig. 1). This may be a consequence of the high overall GC content of these ITS2 sequences. Changes in CAG repeat copy number could lead to alternative ITS2 intramolecular folding structures that would still be rich in GC pairs, and thus retain the low-energy folding pattern that appears to be a characteristic of this region (Wesson et al. 1992, Fritz et al. 1994).

If this CAG repeat and its flanking regions are excluded from comparisons, species A and B differ by only ≈7%, whereas B and D differ by ≈14% (precise estimates of difference depend on region excluded). These numbers are similar to those reported in the An. hermsi, An. freeborni, and An. occidentalis ITS2 comparisons. Although An. occidentalis has morphological features that allow it to be distinguished from An. hermsi and An. freeborni, it is able to form fertile female hybrids with both of the latter 2 species and thus may be no more evolutionarily diverged than An. quadrimaculatus species B and D.

One obvious consequence of the type of interspecific variation observed in the An. quadrimaculatus complex ITS2 sequence comparisons is that the concentration of species-specific differences in the CAG repeat region complicates the use of this region for drawing inferences about the phylogenetic relationships among members of this complex. This caution probably should be extended to the use of this region for phylogenetic inference in general, particularly when species-specific differences are clustered in regions with subrepeat structure.

Polymerase Chain Reaction Assay. The nucleotide conservation of the 3' end of the 5.8S and differences in the sequences of the ITS2 region of the members of the An. quadrimaculatus complex have enabled us to develop a PCR assay to separate 4 of the 5 members of the complex. The small differences between the ITS2 rDNA sequences of species C1 and C2 were insufficient for selection of primers that would separate these 2 species. Species C1 and C2 produced bands of equal size which differed from those of the other 3 species in the complex. The sizes of the bands that separated species A from C1 and C2 differed by only 26 bp, so we recommend that the gel be run until the sylene cyanole in the loading dye has migrated at least 4 cm. Although this assay cannot be used to distinguish C1 from C2, it should be possible to distinguish between these 2 species by a PCR assay based on a pair of primers that flank the CAG repeats. Species C1 has 10 repeats, whereas C2 has only 6, a difference in length of 12 bp. Although

References


Collins, M. A. Mendez, N. B. Besansky, A ribosomal RNA gene pro
this length difference would be difficult to resolve on a standard agarose gel, there are other types of gels that easily can resolve such length differences. Some members of the An. quadrimaculatus complex have been the subject of many ecological, behavioral, disease vector competence, and genetic studies because of their nuisance and potential medical importance, abundance, and ease with which they can be colonized. A quick and reliable technique for distinguishing the members of the complex would therefore be a useful tool. In laboratories set up for PCR reactions as many as 100 specimens per day can be identified by a single individual. PCR-based diagnostic assays are nonradioactive and applicable to all life stages. Furthermore, rDNA genes are highly repeated, and thus small amounts of DNA from poorly preserved specimens generally contain enough intact template to enable species identifications (Scott et al. 1993). Because DNA from a single leg may be sufficient for a PCR identification, the species identity can be determined without killing the specimen. The use of species-specific DNA probes such as that developed by Johnson et al. (1993) for the identification of An. quadrimaculatus species A could be adapted for field use, but until such probes are available for all species in the complex, this PCR diagnostic assay may be the best available option.

This PCR assay thus far has been used only on specimens from the populations that formed the basis of this study, all of which were from Florida. At least 10 different cytogenetically identified individuals from each population were tested, and all produced the expected diagnostic PCR fragment. Work is in progress to verify the specificity of this PCR diagnostic assay with cytologically and electrophoretically identified specimens of the An. quadrimaculatus complex collected from other parts of their distributions.

Acknowledgments

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ABSTRACT The Mississippi Valley fever virus (serotype 2) of the genus *Pseudotogavirus* is a positive contrast agent for white-tailed deer with viremia. Titrations of EHDV-2 ingested in deer with viremia of EHDV-2 in deer with viremia of 10^10^ TCID<sub>50</sub> ml<sup>-1</sup> may be a cause of disease in cattle. The vectors of EHD are midges of the genus Culicoides (Coquilletti) in domestic ruminants with EHD viruses occasional as a cause of disease in cattle. The authors report a positive contrast agent for white-tailed deer with viremia of EHDV-2 in deer with viremia of 10^10^ TCID<sub>50</sub> ml<sup>-1</sup> may be a cause of disease in cattle. The vectors of EHD are midges of the genus Culicoides (Coquilletti) in domestic ruminants with EHD viruses occasional as a cause of disease in cattle.