**Characterization of five unclassified orthobunyaviruses (Bunyaviridae) from Africa and the Americas**

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**Abstract**

The *Bunyaviridae* family is made up of a diverse range of viruses, some of which cause disease and are a cause for concern in human and veterinary health. Here, we report the genomic and antigenic characterization of five previously uncharacterized bunyaviruses. Based on their ultrastructure, antigenic relationships and phylogenomic relationships, the five viruses are classified as members of the *Orthobunyavirus* genus. Three are viruses in the California encephalitis virus serogroup and are related to *Trivittatus* virus; the two others are most similar to the *Mermet* virus in the Simbu serogroup, and to the *Tataguine* virus, which is not currently assigned to a serogroup. Each of these five viruses was pathogenic to newborn mice, indicating their potential to cause illness in humans and other animals.

**INTRODUCTION**

The family *Bunyaviridae* is a large and diverse group of RNA viruses, infecting vertebrates, arthropods and plants. The bunyavirus genome consists of three unique molecules of negative or ambisense ssRNA, designated L (large), M (medium) and S (small), which total 11–19 kb [1]. Viruses in the family *Bunyaviridae* are classified into five genera, *Orthobunyavirus*, *Nairovirus*, *Hantavirus*, *Phlebovirus* and *Tospovirus*. The terminal sequences of genome segments are conserved among viruses in each genus, but are different from those of viruses in other genera.

The largest genus within the *Bunyaviridae* family is the *Orthobunyavirus* genus. Most orthobunyaviruses are mosquito-borne, but several are transmitted by ticks, phlebotomine sandflies and culicoid midges. The demarcation of *Orthobunyavirus* species has proved difficult because of the lack of biochemical characterization of many of the named virus isolates [1]. Consequently, species within this genus have primarily been defined by antigenic criteria, using haemagglutination inhibition (M segment-determined), complement fixation (S segment-determined) and neutralization (M segment-determined) tests. The ability of bunyaviruses to undergo segment reassortment in nature [2–6] has confounded their antigenic classification, since these serological tests only react with a single genome segment. Reassortment among related viruses can also introduce major phenotypic changes caused by new combinations of segments [2], so a more precise identification is needed. For this reason, full-genome sequencing with comparison of phylogenetic relationships based on all three segments can complement serological- and ecological-based methods for more accurate identification of uncharacterized bunyaviruses.
bunyavirus classification. Unfortunately, full-genome sequences are still unavailable for many of the named bunyaviruses [1, 7]. As part of a larger project of arbovirus discovery, this paper describes the genetic, antigenic and ultrastructural characteristics of five previously unclassified orthobunyaviruses.

RESULTS

Serological results

Two of the orthobunyaviruses included in this study were strains of named viruses that have already been characterized antigenically [8]. Virus strain TX-809 was isolated in 2002 from a dead black-and-white warbler (Mniotilta varia) in Harris County, Texas, USA, during West Nile virus surveillance studies. Serological studies performed at the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA) at that time indicated that it was a strain of Mermet virus (MERV) (data not shown). MERV occurs in the midwestern United States and has been associated with birds and Culex mosquitoes [8]. MERV is included in the Simbu serogroup of orthobunyaviruses, based on its antigenic relationships. Virus strain IBH10362 was originally isolated from a febrile child in Ibadan, Nigeria in 1966. Based on serological tests performed at the time, it was identified as Tataguine virus (TATV), an ungrouped orthobunyavirus that has been associated with febrile illness in people in West Africa [9].

Virus strains PanAr 395145 and PanAr 395144 were isolated from Aedes mosquitoes in Panama. Based on serological tests performed at the WRCEVA (Table 1), these two virus isolates are indistinguishable and appear to be related to, but distinct from, Trivittatus virus (TVTV), a member of the California serogroup. We propose the name ‘Achiote virus’ (ACHOV) for this novel agent.

Virus strain Tampa 08 is the prototype strain of a California serogroup virus, designated Infirmatus virus (INFV). Tampa 08 was isolated from a pool of Aedes infirmatus mosquitoes collected in 2008 in Tampa Bay Downs, a thoroughbred horse-racing facility in Hillsborough County, Florida, USA. Although no serology was performed initially, partial sequencing and comparative analysis (by BLASTN) against NCBI nucleotide databases indicated that Tampa 08 virus was related to TVTV [10]. A total of 13 isolates of INFV were made at the same site in 2008 from pools of 4 different mosquito species: 2 from Ae. infirmatus; 1 from Anopheles crucians; 8 from Culex nigripalpus; and 2 from Cx. quinquefasciatus [10]. Complement fixation (CF) serological studies performed at the WRCEVA confirmed that INFV is a member of the California encephalitis serogroup and related to the 2 ACHOV isolates, PanAr 395144 and PanAr 395145, in this study, as well as to TVTV (Table 1).

Transmission electron microscopy

The virions of strains IBH10362 and PanAr 395144 had diameters of about 75 nm and were localized to the vacuoles in the Golgi region, which were probably Golgi-derived, as observed in ultrathin sections of infected BHK cells (Fig. 1a, b, d, e). The virions of TX-809 virus were 85–100 nm in diameter and were observed in clusters at the cell surface in infected Vero E6 cells (Fig. 1c).

Growth in cell culture and pathogenicity for CD-1 mice

Mermet virus (MERV)
The TX-809 strain of MERV produced massive cytopathic effect (CPE) in Vero E6 cell cultures within 3 days. It also caused illness on day 3 in newborn mice after intra-cerebral (IC) inoculation. It was not pathogenic to adult mice after intraperitoneal (IP) injection, and they subsequently developed antibodies.

PanAr 395145 and PanAr 395144 (ACHOV)
PanAr 395145 produced massive CPE in both BHK and Vero E6 cells 2 days after inoculation. In contrast, virus strain PanAr 395144 took 9 days to produce comparable CPE in Vero E6 cells; it was not tested in BHK cells. After IC inoculation of newborn mice, PanAr 395145 produced severe illness and death within 5 days and PanAr 395144 produced illness in 3 days. Neither virus caused illness in adult mice after IP inoculation, but both groups of mice subsequently developed CF antibodies.

Infirmatus virus (INFV)
Inoculation of INFV strain Tampa 08 into Vero E6 cell monolayer cultures produced massive CPE within 4 days. Newborn mice inoculated with the virus by the IC route were all moribund or dead within 3–4 days. Adult CD-1 mice (n=5) inoculated IP with the virus did not show signs of illness, but all developed specific CF antibodies post-infection.

Tataguine virus (TATV)
Strain IBH10362 of TATV produced CPE in Vero E6 cells in 8 days, and death in newborn mice 5–6 days after IC inoculation. Adult mice showed no signs of illness but developed CF antibodies after IP injection.

Genome organization

The three segments in each virus encode uninterrupted open reading frames for the L protein (segment L), the M polyprotein (segment M) and the nucleocapsid protein (segment S) (Table 2). No additional genes were identified by searching NCBI protein databases with the translated nucleotide sequences (BLASTX). It is, however, possible that an additional non-structural protein failed to assemble on the S segments downstream of the nucleocapsid protein, as this is a common feature of other orthobunyavirus genomes. For some segments, the 5’ and 3’ ends of the molecules are missing as a result of the random priming PCR and sequencing approach employed. In 5 of the 12 segments, this resulted in a truncation of a protein-coding region. No attempts were made to recover the 5’ and 3’ ends.

MERV strain TX-809 shows 99–100% amino acid identity to the recently published sequences of the L, M and S proteins of the MERV prototype, AV782 [3] (Table S1,
Table 1. Results of complement fixation tests comparing PanAR 395144 and PanAR 395145 antigens with antibodies to the homologous viruses and to other California encephalitis serogroup viruses

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*Titre is reported as the reciprocal of highest antibody dilution over highest antigen dilution. Zeros indicate a value <4/4.

CE, California encephalitis; INF, Infirmatus viruses; INK, Inkoo; JC, Jamestown Canyon; KEY, Keystone; LAC, La Crosse; LUM, Lumbo; MEL, Melao; SDN, Serra do Navio; SSH, Snowshoe hare; TAH, Tahyna; TVT, Trivittatus

Lighter shade of gray: serologic relationships of the two PanAr viruses against all antigens tested (first column). Darker shade of gray: serologic relationships of the two PanAr viruses against TVT and INF viruses.

available with the online Supplementary Material). TX-809 also shows high nucleotide identity (96–98%) to the published MERV L, M, and S segments. The ACHOV PanAr virus strains are most similar to TVTV at the amino acid level for all three proteins. The two ACHOV PanAr virus strains are most identical at the nucleotide level to TVTV in both the L and S segments, but differ in their identity in the M segment. The PanAR 395144 M segment has the highest nucleotide identity to LaCrosse virus, whereas the PanAR 395145 M segment is most identical to CEV strain BIS283. Taken together, these results confirm the serology results, which identified these two strains as members of the California encephalitis serogroup. INVF is most similar to TVTV across all segments and proteins. IBH10362 has 99% identity at the nucleotide and amino acid level to the published TATV sequence for all three segments [11] (Table S1).

Phylogenetic analysis

We reconstructed maximum-likelihood trees from orthobunyaviruses for each individual segment due to large differences in character number and sampling across the L, M, and S proteins (Table S2). A global orthobunyavirus tree (Fig. 2) and subtrees of the California and Simbu serogroups (Fig. 3) display both the position of these viruses within the context of the Orthobunyavirus genus and the finer scale relationships within California encephalitis and Simbu serogroups (to which four of these five viruses belong), respectively.

Trees for the L, M and S proteins recapitulate the higher order rankings observed in their respective phylogenies, grouping TX-809 with MERV in the Simbu serogroup in all three trees (Fig. 2). The absence of molecular data from more closely related Simbu viruses prevents further comparison. The ACHOV PanAr viruses and INFV were grouped in the California encephalitis serogroups in all three trees (Fig. 2). Unlike the other four viruses, IBH10362 does not group clearly with any of the other well-sampled serogroups of orthobunyaviruses. It does, however, branch with robust support at the base of the Anopheles A and B serogroups, along with TATV in the S tree (Fig. 2). IBH10362 fails to fall within any of the larger orthobunyavirus serogroups in both the L and the M protein phylogenies, and in the absence of corresponding sequences for members of the Anopheles A or B serogroups, the basal relationships of TATV strain IBH10362 to these groups is not observed.
The nucleocapsid protein tree encoded by the S segment indicates that the MERV strain TX-809 branches at the base of a clade of Manzanilla complex viruses in the Simbu serogroups (Fig. 3). This tree is equivocal in its support due to two other very similar viruses, Ingwavuma and Manzanilla. Nevertheless, it is clear that TX-809 is more similar to the MERV prototype virus by branch length in the M and L phylogeny, and at the level of amino acid identity, than it is to the Ingwavuma and Manzanilla viruses. It shows 99% identity to the published MERV in the L segment compared to 92% for Ingwavuma and 91% for Manzanilla; it also has 99% identity to the published MERV in the M segment compared to 83% for Ingwavuma and 88% for Manzanilla.

PanAr 395145 and PanAr 395144 branch with TVTV in the nucleocapsid protein tree (Fig. 3). Also branching within the TVTV complex, INFV branches closely to the ACHOV PanAr viruses (Fig. 3). This clade of Trivitattus-like viruses appears as a long branch within the California encephalitis serogroup and clusters within the Melao complex of the California serogroup, making the Melao complex paraphyletic in the S segment tree. This relationship is poorly supported and this topology is not recovered in the reconstruction of the L and M protein trees. The ACHOV PanAr viruses branch with TVTV at the base of the California encephalitis clade in the M polyprotein phylogeny, while the Melao virus falls deeper within the California clade, as seen in Fig. 3. The L protein phylogeny also places the ACHOV PanAr viruses at the base of the California encephalitis serogroup (Fig. 3). INFV branches as close sister to TVTV and the ACHOV PanAr viruses in both the L and M trees (Fig. 3).

DISCUSSION

The five viruses described in this study vary greatly in their host range, geographical distribution and human disease potential. MERV has been isolated from *Culex* mosquitoes and a variety of bird species from the central United States, but it has not yet been associated with human illness or...
The two Panamanian viruses, PanAr 395144 and PanAr 395145, were isolated from two pools of *Aedes* mosquitoes collected in Panama in the 1970s. While they are indistinguishable antigenically, they are clearly members of the California serogroup. The human disease potential of these strains is unknown. Likewise, to date, all isolates of INFV have been obtained from mosquitoes, so its vertebrate hosts and disease potential are also unknown. However, since a number of the California encephalitis serogroup viruses are known human pathogens [12, 13], we assume that INFV and ACHOV have the potential to infect humans and cause illness. TATV has been isolated from a variety of mosquito genera and species, and from humans in Senegal, Cameroon, Nigeria, Central African Republic and Ethiopia [8]. Clinical cases of human infection with TATV have been characterized by a self-limited febrile illness with headache, rash and joint pains [9].

Recent molecular studies show that the California encephalitis serogroup viruses can be divided into three complexes: California encephalitis, Melao and Trivittatus [14, 15]. Our phylogenetic studies of both the L and M proteins split these three complexes within the California serogroup, with INFV and the ACHOV PanAr virus isolates branching at the base with TVTV. The phylogeny of the nucleocapsid protein (encoded by the S segment) recovers this same relationship with TVTV, but here the support for the monophyly of the Melao clade is low. Further, the Melao clade is paraphyletic in the nucleocapsid protein phylogeny, as it includes the divergent Trivittatus clade. This lack of resolution in the nucleocapsid protein phylogeny vis-a-vis the L and M trees could be a result of the smaller size of the nucleocapsid protein and a reduced number of characters from which to draw the trees. In spite of conflicting topologies within the

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**Fig. 2.** Maximum-likelihood phylogenetic analysis performed on the sequence of the L (polymerase), M (glycoprotein) and S (nucleocapsid) proteins. The sequences reported in this paper are displayed in larger blue text. Likelihood bootstrap support values where ML support is greater than 50% are shown above nodes. For presentation, bootstrap and taxon names at internal nodes within well-sampled serogroups have been deleted. See Fig. 3 for complete taxon names and support values for the relevant California encephalitis and Simbu serogroups.
Fig. 3. Subtrees of the Simbu and California encephalitis serogroups, within which four of the reported viruses branch. Trees for each peptide (L, M and S) are displayed along rows. Simbu and California encephalitis serogroups are displayed along columns. ML bootstraps are shown at nodes, where bootstrap support is greater than 50% for the relationship.
Trivittatus group, it is clear from all three trees that the ACHOV virus strains share a closer common ancestor with TVTV in the phylogeny than they do with the Melao serogroup. Taken together, this shows that phylogenetically distinct California serogroup strains may still be antigenically similar, but that closely related strains may be antigenically distinct. The classification of virus strains on the basis of serological testing alone should thus be treated with caution, since the CF and neutralization tests only react with a single genome segment.

Sequence identity comparisons alone have proven to be unable to predict a serogroup for the IBH10362 (TATV) virus, and here phylogenetic analysis assists in reconstructing its position within the orthobunyavirus serogroups. Phylogenies of the L, M and nucleocapsid (S) proteins all fail to place IBH10362 within any of the major serogroups, and also fail to recover a consistent relationship between IBH10362 and the serogroups. The L phylogeny places IBH10362 as a sister to the CEV, while the M phylogeny recovers a sister relationship to the CEV, Bunyamwera and Wyeomyia serogroups. The nucleocapsid tree, which is the most comprehensively sampled of the three, recovers strong support for a sister relationship between IBH10362 and Anopheles serogroups A and B.

The addition of newly sequenced complete orthobunyavirus genomes can have a significant impact on the phylogeny of this genus. At the time the strains reported here were sequenced, the L and M segments of the MERV were not yet available, so the TX-809 strain was initially found to be most similar to the Oropouche virus. The subsequent publication of the complete MERV genome [3] allowed for a more thorough analysis, which indicated that the highest similarity for all segments of TX-809 is to MERV. The IBH10362 virus strain was also initially identified as TATV based on serology; however, at the time of sequencing the L, M and S segments showed the highest amino acid similarity to the Chatanga virus, Keystone virus and Tacaiuma virus, respectively. The recent publication of the TATV H9963 genome has now allowed IBH10362 to show similarity at the nucleotide and amino acid levels with TATV, in addition to at the level of serology. Therefore it will be important to keep updating the segment phylogenies and genome relationships of this genus as newly sequenced genomes become available.

We have performed genome scale analyses on five members of the Orthobunyavirus genus. Through a combination of phylogenetic analyses, electron microscopy and serological testing, we can assign four of these five viruses to existing orthobunyavirus serogroups, and demonstrate that one of them, Tataguine virus, may represent a novel serogroup in the Orthobunyavirus genus. We also demonstrate that two of the viruses, Infirmatus and Achiote, are closely related to Trivittatus virus, and thus form a third and expanded complex within the California encephalitis serogroup.

METHODS

Viruses

The five viruses examined in this study were obtained from the WRCEVA at the University of Texas Medical Branch. The available information on their origin, date of isolation and locality is given below.

- Mermet (strain TX-809). This virus was isolated at the WRCEVA from the brain of a dead black-and-white warbler (Mniotilta varia) collected on 11 October 2002 during a West Nile virus surveillance program in Harris County, Texas, USA.
- Achiote (strains PanAr 395144 and PanAr 395145). These two viruses were isolated from mosquito pools of Aedes mosquitoes collected and processed by the Gorgas Memorial Laboratory, Panama City, Panama in the period from 1973 to 1979. The exact collection site(s) and dates or species composition of the pools are not available, but the viruses were deposited in the WRCEVA collection as unknown viruses in 1980.
- Infirmatus (strain Tampa 08). This virus was originally isolated from a pool of Aedes infirmatus mosquitoes collected in Tampa, Florida, USA by a group from the University of South Florida on 22 April 2008 during an investigation of an equine case of encephalitis.
- Tataguine (strain IBH10362). This virus was isolated from the blood of a febrile child visiting an outpatient paediatric clinic at the University of Ibadan, Ibadan, Nigeria on 15 June 1966 [16].

All viruses were propagated in Vero E6 or BHK-21 cells (originally obtained from the American Type Culture Collection, Manassas, VA, USA) in preparation for ultrastructural and sequencing studies.

Transmission electron microscopy

For ultrastructural analysis in ultrathin sections, infected BHK or Vero E6 cells were fixed for at least 1 h in a mixture of 2.5 % formaldehyde prepared from paraformaldehyde powder, and 0.1 % glutaraldehyde in 0.05M cacodylate buffer (pH 7.3) to which 0.03 % picric acid and 0.03 % CaCl2 were added. The monolayers were washed in 0.1 M cacodylate buffer and cells were scraped off and processed further as a pellet. The pellets were post-fixed in 1 % OsO4 in 0.1M cacodylate buffer (pH 7.3) for 1 h, washed with distilled water and en bloc stained with 2 % aqueous uranyl acetate for 20 min at 60 °C. The pellets were dehydrated in ethanol, processed through propylene oxide and embedded in Poly/Bed 812 (Polysciences, Warrenton, PA, USA). Ultrathin sections were cut on Leica EM UC7 ultramicrotome (Leica Microsystems, Buffalo Grove, IL, USA), stained with lead citrate and examined in a Philips 201 transmission electron microscope at 60 kV.

Serological tests

CF tests were performed by the microtitre technique [17], using 2 units of guinea pig complement and overnight incubation of the antigens and antibodies at 4 °C. The antigens
used in the CF tests were prepared from infected newborn mouse brain by the sucrose acetone extraction method [18] and inactivated with 0.05% β-propiolactone (Sigma, St Louis, MO, USA) or by gamma irradiation. CF titres were recorded as the highest dilutions giving 3+ or 4+ fixation of complement on a scale of 0 to 4+.

**Antigens and antisera**

The antisera for serological tests were prepared in adult mice, using 10% crude homogenates of infected newborn mouse brain in phosphate-buffered saline as the immunogen [5]. The immunization schedule consisted of four intraperitoneal injections of antigen mixed with Freund’s adjuvant, given at weekly intervals. After the final immunization, mice were inoculated with sarcoma 180 cells, and the resulting immune ascitic fluids were collected. All animal work was performed at the University of Texas medical branch (UTMB) under an UTMB IACUC-approved animal use protocol (number 9505045).

**Virus preparation for sequencing**

Vero E6 cell-amplified viruses were purified before RNA extraction. Samples were first pre-cleared by low-speed centrifugation at ~3200 g for 5 min. The supernatants were spun through 0.45 µm filters to remove eukaryotic cells. Samples were then concentrated with a Vivaspin 20 column (Sartorius) and treated with 2U Turbo DNase (Ambion), 1U Benzonase (Novagen) and 2U Rnase One (Promega) for 1 h at 37 °C. Finally, 2 µl of GlycoBlue (Invitrogen) was added and the viruses were pelleted through a 10/15/20% sucrose gradient by spinning at 39000 r.p.m. for 1.5 h. Viral pellets were resuspended in nuclease-free water and the presence of virus was confirmed by EM. The pelleted virus was lysed and the genomic material purified using Trizol (Invitrogen) or the Viral RNA mini kit (Qiagen).

**Next-generation sequencing**

The extracted viral RNAs were reverse transcribed and tagged using the SISPA methodology [19]. Briefly, 4–9 µl of viral RNA was reverse transcribed in a mix of 1 µM tagged random primers, 3% DMSO, 10 mM DTT, 500 µM dNTPs, 8U RNase OUT (Invitrogen) and 100U Superscript III (Invitrogen) at 25 °C for 10 min, 50 °C for 50 min and 85 °C for 10 min. Second-strand synthesis was performed with 2.5U of 3’–5’ exo-Klenow polymerase (NEB) at 37 °C for 60 min and 75 °C for 10 min. The reactions were treated with 1U shrimp alkaline phosphatase (Fisher) and 2U exonuclease I (USB) at 37 °C for 60 min and 72 °C for 15 min. Final PCR amplification was performed on 4 µl of tagged cDNA with 1U Accuprime Taq Hifi (Invitrogen) and 500 µM tag primer in buffer I using a cycle with initial denaturing at 94 °C for 2 min, then 25–30 cycles of 94 °C for 30 s, 55 °C for 30 s and 68 °C for 30–60 s, and then a final extension of 68 °C for 10 min. The PCR products were visualized by gel electrophoresis and fragments from 200 to 400 bp gel were purified using a MinElute gel extraction kit (Qiagen). A multiplex sequencing pool was built using equal amounts of each tagged DNA and purified with a MinElute PCR kit (Qiagen). The pool was end-repaired and sequence adapter-ligated. Sequencing was performed on the Illumina HiSeq2000 with 100 bp paired-end chemistry.

**Sequence assembly**

Demultiplexed reads were quality trimmed and assembled with the Velvet de novo assembler [20], using a range of k-mer values between 33 and 63. Additionally, the expected coverage values estimated from the library size and contig k-mer coverage of previous assembly attempts were used to optimize assembly. The trimmed reads of PanAr 395144 were mapped to the PanAr 395145 genome, and subsequently assembled using the Velvet Columbus module. The quality of each assembly was assessed on the basis of the N50 and maximum contig size, and each was searched by BLASTX against a local version of the nr database limited to viral accessions. The top BLASTX hits were used to identify closely related viral genomes, and these were then used as references to order assembled contigs using the ABACAS.pl script [21].

Gaps in assembled contigs or between ordered contigs were closed by designing primers using Primer3 software. Amplions of 500–1000 were then generated with the Accuprime Taq Hifi kit (Invitrogen) and purified with the MinElute PCR purification kit (Qiagen). Sanger sequencing of the amplicons was conducted from both the forward and reverse directions.

**Phylogenetic analysis**

A broadly representative group of orthobunyavirus sequenc- es was retrieved from GenBank by BLASTX matches to the newly sequenced viruses. These were then aligned using MUSCLE v3.8.31 [22], and the resulting multiple sequence alignments were inspected in the Seaview multiple alignment viewer [23]. Character sets were initially selected for phylogenetic analyses using Gblocks, and then manually adjusted by eye [24].

Phylogenetic trees of orthobunyaviruses (Table S2) were generated for each protein (S, M and L) with RaxmlHPC using the protein GTR substitution model, with rates across the sites being modelled on a gamma distribution with invariant sites. One thousand bootstrap replicates were performed for each tree. An alignment of 39 taxa and 1520 characters was used in downstream L protein analyses, with 57 taxa and 1091 characters being used in the M polyprotein phylogeny and 82 taxa and 223 characters being used for the nucleoprotein phylogeny.

**Funding information**

This work was supported in part by NIH contract HHSN272201000040I/ HHSN2700004/D4 (NV, RBT) and grant R24AI120942 (NV, RBT).

**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**Ethical statement**

All animal work was performed at the University of Texas Medical Branch under Animal Care and Use Protocol number 9505045.
References


