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Reptiles and Amphibians as Potential Reservoir Hosts of Chikungunya Virus

Angela M. Bosco-Lauth,* Airn E. Hartwig, and Richard A. Bowen
Department of Biomedical Sciences, Colorado State University, Fort Collins, Colorado

Abstract. Chikungunya virus is an emerging arbovirus of significant human-health concern. Little is known about its sylvatic cycle, including whether ectothermic vertebrates are permissive to infection. In this study, individuals from ten species of reptiles and amphibians were inoculated with chikungunya virus and samples of blood were tested to characterize viremia and seroconversion. Viremia was not detected in cane toads, house geckos, or American alligators, but most of the green iguanas, red-eared sliders, ball and Burmese pythons, leopard frogs, Texas toads, and garter snakes developed viremia. Peak virus titers in serum of up to 4.5, 4.7, and 5.1 $\log_{10}$ plaque-forming units per milliliter were observed for garter snakes, ball pythons, and Texas toads, respectively. These results add to those of other studies that have suggested a possible role for ectothermic vertebrates in the ecology of arbovirus maintenance and transmission in nature.

INTRODUCTION

Chikungunya virus (CHIKV; *Togaviridae: Alphavirus*) is a mosquito-borne virus of high medical importance that was first recognized as a human pathogen in Tanzania in 1952.[1,2] The virus is broadly distributed throughout Africa and Asia, and recently expanded to become endemic in large regions of Central and South America.[3,4] Epidemic transmission is primarily through a human–mosquito–human cycle involving *Aedes aegypti* and *Aedes albopictus* mosquitoes.[5] The virus is thought to be maintained in nature by circulation in arboreal primates,[5,6] although this sylvatic transmission cycle is poorly understood and other species may be involved as reservoirs.

The possible role of ectothermic vertebrates as reservoirs or overwintering hosts has been evaluated for several arboviruses, and numerous species of mosquitoes have been described to feed on a variety of reptiles and amphibians, including such anthropophilic mosquitoes such as *Ae. aegypti*.[7] Serological surveys from decades ago revealed antibodies to a variety of arboviruses in sera from turtles, tortoises, snakes, lizards, and frogs.[8–22] Moreover, blood meal identification in mosquitoes has demonstrated that ectothermic hosts may play an important role in supporting these vectors.[12–14]

The two best studied cases for ectothermic vertebrates as hosts of arboviruses involve eastern equine encephalitis viruses (EEEVs) and western equine encephalitis viruses (WEEVs). In the case of EEEV, several species of mosquitoes collected in an endemic area have been shown to harbor virus, and blood meal analysis indicated that they commonly fed on ectotherms, including cottonmouth snakes and frogs.[12] Moreover, sera from both cottonmouth and copperhead snakes were positive for viral RNA, and inoculation of garter snakes with EEEV resulted in viremia of substantial magnitude; inoculated anoles also developed viremia, but of a low titer, and frogs did not become detectably viremic.[15,16] Importantly, some garter snakes were induced to enter brumation 2 days after virus inoculation, maintained at 7°C for 30 days, and were found to develop high titer viremia on exit from brumation.[16] In the case of WEEV, viremia was detected in both garter snakes and frogs collected in the field.[9] Garter snakes infected with WEEV and placed in a hibernaculum were able to maintain the virus in blood for roughly 6 months; and, after emergence, a substantial majority of snakes developed viremia of up to 6.3 $\log_{10}$ that lasted up to 70 days.[17] *Culex tarsalis* mosquitoes that fed on the snakes after emergence became infected and were able to transmit the virus to chicks. Certainly, this points to a potential mechanism for overwintering of WEEV. In a follow-up study, *Cx. tarsalis* mosquitoes infected with WEEV were able to transmit virus to garter snakes; the snakes were again able to maintain the virus for months in brumation and developed viremia after emergence.[18] Finally, Texas tortoises are yet another reptile shown to develop a high-titered and prolonged viremia after experimental infection with WEEV.[19] In addition to these two alphaviruses, three flaviviruses—Japanese encephalitis, West Nile, and Zika viruses—have been shown to replicate in reptiles and amphibians.[20–24]

In a previous study, we experimentally inoculated a variety of mammals and birds, and failed to identify hosts likely to serve as reservoirs of CHIKV, with the possible exception of bats.[25] Here, we report on a series of experiments designed to identify potentially competent host species among reptiles and amphibians.

METHODS

Viruses. Two isolates of CHIKV were obtained from the Centers for Disease Control and Prevention, passaged once in Vero cells and frozen at –80°C in Dulbecco's Modified Eagle Medium and 10% fetal bovine serum (FBS). SAH (South African Human) isolate was obtained from a human in South Africa in the 1970s. COM (Comoros) is an isolate from a human from the Comoros Islands outbreak in 2005. Both isolates are representatives of the Central/East African clade.

Animals. The animals tested for susceptibility to CHIKV infection are listed in Table 1. All animals were purchased from biological supply companies or from private individuals and housed under biosafetly level 3 containment at Colorado State University. Snakes were housed in glass aquariums and provided a heat source and a source of ultraviolet (UV) light as needed. Frogs, toads, and geckos were housed in plastic aquariums and provided heat and UV light. Alligators, red-eared sliders, and iguanas were housed in 200-gallon metal stock tanks. The alligators and turtles were allowed access to "sunning" via a ramp out of the water that was positioned

* Address correspondence to Angela M. Bosco-Lauth, Department of Biomedical Sciences, Colorado State University, Fort Collins, CO 80523. E-mail: angela.bosco-lauth@colostate.edu

TABLE 1

Viremia and neutralizing antibody responses in reptiles and amphibians inoculated with chikungunya viruses

Host species	Number viremic/number tested (range in peak viremia, $\log_{10}$ PFU/mL)	Number seropositive at 28 DPI/number tested ($PRNT_{80}$ titer range)
Ball python *Python regius*	6/8 (2.8–4.3)	7/8 (10–320)
Burmese python *Python bivittatus*	3/4 (2.3–3.0)	4/4 (80–320)
Garter snake *Thamnophis sirtalis*	6/6 (3.7–4.5)	5/5 (20–80)
Leopard frog *Lithobates* spp.	20/20 (3.0–4.7)	0/20*
Texas toad *Anaxyrus speciosus*	7/12 (2.3–5.1)	11/12 (20–40)
Cane toad *Rhinella marina*	0/4	4/4 (80–320)
American alligator *Alligator mississippiensis*	0/6	6/6 (20–160)
Green iguana *Iguana iguana*	6/7 (2.0–3.7)	3/4 (20–80)
House gecko *Hemidactylus* spp.	0/7†	Not tested
Red-eared slider *Trachemys scripta*	4/6 (2.3–3.7)	3/6 (10–80)

DPI = day postinoculation; PFU = plaque-forming units; PRNT = plaque reduction neutralization assay.

* Frogs were euthanized and tested for antibody at 21 DPI rather than 28 DPI.

† Geckos were terminally bled on 2, 3, 4 DPI ($N = 2$), and 6 ($N = 1$) DPI.

beneath heat and UV bulbs. The iguanas were provided newspaper as bedding, limbs for enrichment, and heat/UV light. All animals were fed according to the prescribed diets from before purchase. Clean water and fresh bedding were supplied as needed. All animal procedures were performed in accordance with the approval from the Institutional Animal Care and Use Committee at Colorado State University.

Virus inoculation and sample collection. Virus stocks were diluted in phosphate buffered saline to achieve desired titers. For most species, half of the animals were infected with COM and half with SAH strains, the exceptions being geckos and garter snakes, which were only infected with SAH because of the small number of animals and inability to sample on consecutive days. All infections were performed via subcutaneous needle inoculations of 0.1 mL, generally in a hind limb for animals with appendages or beneath the dorsal scales approximately mid-body of the snakes. The inocula were titrated either immediately postinfection or after freezing with 10% FBS. The day of inoculation was considered day 0, and sample collection started 1 day postinoculation (DPI). Because of the differing animal size, blood sample volume, and anatomic location, the frequency of sampling varied among species. Blood was collected daily for 5 days following inoculation for alligators, ball pythons, Burmese pythons, iguanas, and sliders, and on alternating days through 8 DPI for garter snakes, leopard frogs, and Texas toads. For blood collection, two house geckos were euthanized on 2, 3, and 4 DPI and one on 6 DPI. Blood samples were collected into serum separator tubes (BD Microtainer; Becton-Dickinson, Franklin Lakes, NJ) and were allowed to sit at room temperature for approximately 30 minutes before centrifuging at 10,000 × *g* and collection of sera, which were frozen at –80°C for later assay. All animals except frogs were euthanized on 28 DPI; frogs were euthanized on 21 DPI. Euthanasia was carried out using pentobarbital overdose followed by cardiac exsanguination.

Virus isolation and titration. Virus isolation was performed using plaque assay on Vero cells as previously described.[7] Briefly, samples were serially diluted in 96 well plates with BA-1 (minimum essential medium [MEM] salts, 1% bovine serum albumin, 350 mg/L sodium bicarbonate, 100 units/mL penicillin, 100 µg/mL streptomycin, 2.5 µg/mL amphotericin B in 0.05 M Tris, pH 7.6) and 0.1 mL aliquots inoculated onto confluent monolayers in six well plates. The samples were adsorbed onto cells for 1 hour at 37°C, shaking the plates occasionally. After adsorption for 45 minutes, an overlay of MEM, 2% FBS, and 0.5% agarose was added, and 48 hours later, a second overlay containing neutral red was added. The plates were then examined for plaque-forming units (PFU), 1 and 2 days after the second overlay. Plaques were picked for each positive animal, frozen in BA-1, and later used for polymerase chain reaction (PCR) sequence confirmation. Briefly, the harvested plaques were thawed at room temperature and inoculated onto Vero cells in six well plates. After the cytopathic effect was evident, RNA was purified from a sample of medium using QIAamp Viral RNA protocol (Qiagen Inc., Valencia, CA) and subjected to reverse transcriptase quantitative PCR using primers 5′ GAY CCC GAC TCA ACC ATC CT and 5′ CAT MGG GCA RAC GCA GTG GTA.[26]

Serology. Terminal serum samples collected from all animals except geckos were heat inactivated at 56°C for 30 minutes and tested for the presence of anti-CHIKV antibodies by using plaque reduction neutralization assay, as previously described.[25] Samples that neutralized ≥ 80% of plaques at a serum dilution of 10 or greater were considered positive.

RESULTS

Clinical disease attributed to virus infection, as determined by abnormal behaviors, anorexia, or mortality was not observed in any of the inoculated animals over the course of the experiments. Three iguanas and one garter snake died following clearance of viremia and were not tested for antibodies. Viremia was detected in individuals from each of the species except alligators, cane toads, and house geckos (Table 1), and there was no apparent difference in the animals inoculated with the SAH versus COM strains of CHIKV. Specifically, the magnitude and duration of viremia and antibody titers were roughly equivalent between the two strains. Viremia persisted for 3–10 days following inoculation, depending on the species. Peak titers typically occurred between 1 and 3 DPI, with most animals clearing infection by 4 DPI. The Burmese pythons, red-eared sliders, and green iguanas all developed short-term viremia, and none of their titers exceeded 4 $\log_{10}$ PFU/mL.

Table 1 summarizes the antibody titers achieved for each group of animals, with the lowest detectable antibody titer of 80% neutralizing at a dilution of 1:10. Most strikingly, the leopard frogs, which had the longest duration of viremia with relatively high titers and 100% infection rate, did not have any detectable antibodies against CHIKV on either 14 or 21 DPI. In addition, none of the red-eared sliders infected with the COM strain of CHIKV developed detectable neutralizing antibodies. By contrast, nearly all other animals, regardless of viremia, had neutralizing antibodies by 28 DPI, independent of the strain of CHIKV used for infection. It should be noted that leopard frogs were euthanized on 21 DPI, whereas most other species were euthanized on 28 DPI; therefore, sampling leopard frogs at a later time point may have revealed seroconversion. House

geckos were not tested for antibodies, as they were not sampled past 7 DPI, which is likely too early for seroconversion to have occurred. It is possible that prolonging the time for which the frogs and geckos were maintained and thus sampled postinfection may have increased the detection of antibodies in those animals.

DISCUSSION

There is substantial evidence that nonhuman primates serve as reservoir hosts for CHIKV in some environments, but relatively little work has been performed to evaluate other potential hosts for this virus. Previous studies from our laboratory indicated that only a small number of common mammals and birds were competent hosts for CHIKV,[25] which prompted us to explore the possibility that reptiles and amphibians are susceptible to infection with the virus and develop a viremia sufficient to potentially infect feeding mosquitoes.

We evaluated individuals from 10 species, including seven reptiles and three amphibians. Most of the species chosen are found in CHIKV endemic areas, whereas the few that are not (garter snakes and Texas toads) serve as ecological equivalents. Our goal was to pick a diverse study set such that we might cover a wide range of potential hosts or close relatives. Viremia was not detected in American alligators, cane toads, or house geckos, but most of the animals from all other species (leopard frogs, toads, ball and Burmese pythons, garter snakes, iguanas, and red-eared sliders) developed viremia following inoculation with CHIKV. None of the inoculated animals developed clinical signs of disease attributable to infection. Given these findings, an important question is whether infected reptiles and amphibians develop viremia titers sufficient to infect feeding mosquitoes. We did not directly address this question, but other investigators have reported infection of *Ae. albopictus* after feeding artificial blood meals containing as little as 3.6 or 3.9 $\log_{10}$ PFU/mL of CHIKV.[27,28] Some animals from each of the species we tested developed a viremia that exceeded this threshold, including ball pythons, garter snakes, leopard frogs, Texas toads, green iguanas, and red-eared sliders, suggesting their potential to amplify CHIKV and participate in virus transmission. Interestingly, the threshold for infection of *Ae. aegypti* with CHIKV has been reported to be considerably higher than that of *Ae. albopictus*, and to our knowledge, none of the other mosquito species known to feed on reptiles and amphibians have been evaluated in this regard.[27–29]

In addition to developing viremia in response to infection, most of the reptiles and amphibians we infected developed neutralizing antibodies against CHIKV after infection. This is useful information, not only because it implies the development of protective immunity in these animals, but also, in a practical sense, because it suggests that reptiles and amphibians could be useful subjects for serosurveillance in endemic areas or border zones.

The investigations described here add to a body of evidence that ectothermic vertebrates may serve as reservoir hosts for a number of vector-borne viruses. Another possible role they could serve in arbovirus ecology is as overwintering hosts in temperate climates. We did not address this possibility as temperature was maintained essentially constant during infection, but other investigators have provided intriguing evidence for virus persistence through a period of brumation in snakes infected with EEEV,[16] or snakes and tortoises infected with WEEV.[17–19]

Our studies provide evidence that a number of reptiles and amphibians develop viremia of sufficient magnitude to infect at least some species of feeding mosquitoes. The key question is whether these animals play a nontrivial role as reservoirs or overwintering hosts for CHIKV. Clearly, additional studies in both the laboratory and field will be required to address this possibility.

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Authors' address: Angela M. Bosco-Lauth, Airn E. Hartwig, and Richard A. Bowen, Department of Biomedical Sciences, Colorado State University College of Veterinary Medicine and Biomedical Sciences, Fort Collins, CO 80523, E-mails: angela.bosco-lauth@colostate.edu, airn.tolnay@colostate.edu, and richard.bowen@colostate.edu.

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