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Initial sequence characterization of the rhabdoviruses of squamate reptiles, including a novel rhabdovirus from a caiman lizard (*Dracaena guianensis*)

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Abstract

Rhabdoviruses infect a variety of hosts, including non-avian reptiles. Consensus PCR techniques were used to obtain partial RNA-dependent RNA polymerase gene sequence from five rhabdoviruses of South American lizards; Marco, Chaco, Timbo, Sena Madureira, and a rhabdovirus from a caiman lizard (*Dracaena guianensis*). The caiman lizard rhabdovirus formed inclusions in erythrocytes, which may be a route for infecting hematophagous insects. This is the first information on behavior of a rhabdovirus in squamates. We also obtained sequence from two rhabdoviruses of Australian lizards, confirming previous Charleville virus sequence and finding that, unlike a previous sequence report but in agreement with serologic reports, Almpiwar virus is clearly distinct from Charleville virus. Bayesian and maximum likelihood phylogenetic analysis revealed that most known rhabdoviruses of squamates cluster in the Almpiwar subgroup. The exception is Marco virus, which is found in the Hart Park group.

Keywords

Rhabdoviridae; phylogeny; *Dracaena guianensis*; Marco virus; Chaco virus; Timbo virus; Sena Madureira virus

Introduction

Rhabdoviruses are enveloped viruses that contain negative-sense single stranded RNA genomes. Together with the families *Paramyxoviridae*, *Filoviridae*, *Bornaviridae*, and a clade containing the genus *Nyavirus*, they form the order *Mononegavirales*

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(Mihindukulasuriya et al, 2009). The family *Rhabdoviridae* is divided into six genera; *Ephemerovirus*, *Lyssavirus*, *Novirhabdovirus*, and *Vesiculovirus* are viruses of animals, and *Cytorhabdovirus* and *Nucleorhabdovirus* are plant viruses (Dietzgen et al, 2012). The genera *Ephemerovirus* and *Vesiculovirus*, together with a number of unclassified rhabdoviruses, form a clade that has been termed the Dimarhabdoviruses, of which all members with known life cycles use arthropod (usually dipteran) hosts at least part of the time (Bourhy et al, 2005; Dacheux et al, 2010).

RNA viruses can mutate rapidly, and comprise the majority of viruses causing emerging infectious diseases (Woolhouse and Gowtage-Sequeria, 2005). As a group, rhabdoviruses cause several diseases of human and veterinary concern, including rabies, bovine ephemeral fever, viral hemorrhagic septicemia, Chandipura encephalitis, and vesicular stomatitis. Many of these are arthropod-borne.

Six rhabdoviruses have been isolated from non-avian reptiles- Marco, Chaco, Timbó, and Sena Madureira viruses from South America (Causey et al, 1966; Tesh et al, 1983, Shope and Tesh, 1987), and Almpiwar and Charleville viruses from Australia (Doherty et al., 1970, Doherty, 1972, Doherty et al., 1973, Monath et al, 1979). The South American viruses were all isolated from one subspecies of teiid lizard, *Ameiva ameiva ameiva*. There has also been one isolation of Chaco virus from another South American teiid, *Kentropyx calcarata*. Chaco virus was isolated from 3/1526 (0.2%) *A. ameiva ameiva*, and 1/4 (25%) *K. calcarata*, implying that the prevalence of virus discovery in *A. ameiva ameiva* may be partially due to investigative bias toward this subspecies. Marco and Chaco viruses have also been found to infect the mosquito *Aedes aegypti* (Causey et al, 1966). Charleville virus has been found in the gecko *Gehrya australis* as well as *Phlebotomus* sp. sandflies and *Forcipomyia* (*Lasiohelina*) sp. midges, and Almpiwar virus has been isolated from the skink *Cryptoblepharus* (*Ablepharus*) *virgatus*. Charleville and Almpiwar viruses have been shown to multiply in experimentally infected mosquitoes (Carley et al, 1973). The isolated rhabdoviruses of non-avian reptiles have not been officially classified beyond the level of the family (*Rhabdoviridae*).

Serologically, Timbó and Chaco viruses have been shown to cross-react with each other but not with Marco virus (Causey et al, 1966, Tesh et al, 1983), and Sena Madureira cross reacts with Timbo and Chaco viruses but not Almpiwar, Charleville, or Marco viruses (Tesh et al, 1983, Calisher et al, 1989). Almpiwar and Marco viruses were not found to cross-react with other rhabdoviruses, and Charleville virus was found to cross-react antigenically with the lyssaviruses (Calisher et al, 1989), as well as Mossuril virus, Kamese virus, Bangoran virus, Flanders virus, and Marco virus (Tesh et al, 1983), of which the first four have recently been recognized as members of a clade tentatively termed the Hart Park group (Dacheux et al, 2010). Limited serologic evidence for vesicular stomatitis virus and Bahia Grande virus infection has been found in non-avian reptiles (Hoff and Trainer, 1973, Kerschner et al, 1986).

However, little sequence characterization of the rhabdoviruses of squamates has been done, consisting of a single report examining the L gene of Charleville and Almpiwar viruses (Bourhy et al, 2005). We are not aware of any reports describing the behavior of these viruses in squamates. Further work is needed to characterize the clinical impact, prevalence, and ecology of rhabdoviruses in squamate reptiles.

Materials and Methods

Samples

A wild-caught caiman lizard (*Dracaena guianensis*, a member of the family Teiidae) imported to the United States from Peru (Jacobson et al., 2001) was examined as part of a routine quarantine protocol in a zoological collection, and blood was drawn for evaluation. Blood smears were stained using Wright-Giemsa staining (Woronzoff-Dashkoff, 2002). Whole blood was fixed in buffered glutaraldehyde and processed routinely for thin-section transmission electron microscopy. Additional samples of whole blood were stored frozen at -80°C .

Timbó virus (isolate BeAn41787), Marco virus (BeAn40290), Chaco virus (BeAn42217), Sena Madureira virus (BeAn303197), Charleville virus (CH9824), and Almpiwar virus (MRM4059) isolates were obtained from the World Reference Center for Arboviruses and Emerging Viruses (WRCAEV) at the University of Texas Medical Branch, Galveston.

PCR amplification and sequencing

RNA was extracted from isolates or blood using the RNeasy Mini Kit (Qiagen, Valencia, CA). Reverse transcription PCR of the RNA-dependent RNA polymerase gene was performed using the OneStep RT-PCR Kit (Qiagen) according to standard protocol using previously described primers PVO3 and PVO4 (Bourhy et al, 2005). To obtain sequence from the caiman lizard rhabdovirus, Two μl of product from the first reaction was used in a second round with primers RepRhabd2F (TWTTYRANGGWYTNACWATGGC) and PVO3. The mixtures were amplified in a thermal cycler (PCR Sprint, Thermo Hybaid) with an initial denaturation at 95°C for 5 min, followed by 45 cycles of denaturation at 94°C for 30 sec; annealing at 45°C for 30 sec, DNA extension at 72°C for 90 sec, and a final extension step at 72°C for 10 minutes. To obtain the 5' end of sequence homologous to the other squamate rhabdoviruses, a different second round with PVO4 and caiman lizard rhabdovirus-specific primer SDZCrev (TGTTTGCCTCTCCAGTTTGA) was run using the same amplification protocol. Attempts were also made to amplify rhabdoviral nucleoproteins using a previously described consensus PCR (Kuzmin et al, 2006).

All PCR products were resolved in 1% agarose gels. The bands were excised and purified using the QIAquick gel extraction kit (Qiagen). Direct sequencing was performed using the Big-Dye Terminator Kit (Perkin-Elmer, Branchburg, NJ) and analyzed on ABI 377 automated DNA sequencers at the University of Florida Center for Mammalian Genetics DNA Sequencing Facilities. All products were sequenced twice in each direction. Primer sequences were edited out prior to further analyses.

Phylogenetic analysis

Predicted homologous 106–145 amino acid sequences of RNA-dependent-RNA polymerase were aligned using MAFFT (Katoh and Toh, 2008). Lettuce big-vein associated virus (GenBank accession number BAC16226), a member of the unassigned non-rhabdovirus genus *Varicosavirus*, was included. Nyamanini virus, a non-rhabdovirus member of the *Mononegavirales* used as an outgroup (Mihindukulasuriya et al, 2009).

Bayesian analyses of amino acid alignments were performed using MrBayes 3.1 (Ronquist and Huelsenbeck, 2003) on the CIPRES server (Miller et al, 2010), with gamma distributed rate variation and a proportion of invariant sites, and mixed amino acid substitution models. Four chains were run and statistical convergence was assessed by looking at the standard deviation of split frequencies as well as potential scale reduction factors of parameters. The first 25% of 2,000,000 iterations were discarded as a burn in.

Maximum likelihood (ML) analyses of each alignment were performed using RAxML on the CIPRES server (Stamatakis et al, 2008), with gamma distributed rate variation and a proportion of invariant sites. The amino acid substitution model with the highest posterior probability in the Bayesian analysis was selected. Bootstrap analysis was used to test the strength of the tree topology (Felsenstein, 1985). Numbers of bootstrap replicates were determined using the stopping criteria by Pattengale et al (2010).

Results

Samples

Pale blue intracytoplasmic inclusions were seen in erythrocytes in Wright-Giemsa stained blood smears of the caiman lizard (Figure 1). These inclusions were further characterized by transmission electron microscopy, and bullet-shaped particles morphologically consistent with rhabdoviruses were seen (Figure 2).

PCR amplification

PCR amplification of Almpiwar virus produced a 408 nucleotide product when primer sequences were edited out. Amplification of Chaco, Charleville, and Timbó viruses produced 411 nucleotide products when primer sequences were edited out. Amplification of Marco virus produced a 405 nucleotide product when primer sequences were edited out. Amplification of Sena Madureira virus produced a 320 nucleotide product when primer sequences were edited out; this sequence did not have the region homologous to the first 91 nucleotides after forward primer PVO4, and several repetitions produced identical results. Amplification of the caiman lizard rhabdovirus with RepRhabd2F and PVO3 produced a 325 nucleotide band after primers were edited out, PVO4 and SDZCrev produced a 206 nucleotide band, and the assembly of the two sequences was 411 nucleotides. Sequences were submitted to GenBank under accession numbers JN882642-JN882648.

Attempts using a previously described consensus PCR to amplify nucleoproteins from any of the rhabdoviruses using squamate hosts were unsuccessful (Kuzmin et al, 2006).

Phylogenetic analysis

The Bayesian tree is shown (Figure 3). Bayesian phylogenetic analysis found that the Blosum62 model of amino acid substitution was most probable with a posterior probability of 0.919, and this was used for ML analysis (Henikoff and Henikoff, 2001). Stopping criteria for ML bootstrapping were reached after 350 subsets. Bootstrap values as percentages from ML analysis are shown on the Bayesian trees (Figure 3).

Discussion

Our phylogenetic analysis placing all known rhabdoviruses of reptiles, including those using squamate and dinosaurian hosts, in the Dimarhabdovirus group is consistent with the theory that they are arboviruses. The probable replication of the caiman lizard rhabdovirus in erythrocytes would also make for efficient transmission to hematophagous insects. Iridoviruses have also been found in erythrocytic inclusions in squamates, and are hypothesized to be arthropod-vectored (Wellehan et al, 2008).

Our Almpiwar virus sequence did not show very strong homology with the first Almpiwar virus sequence found in GenBank (accession # AY854645), showing only 69% nucleotide homology and 77% predicted amino acid homology over the sequenced region. The AY854645 sequence is 100% identical to the Charleville virus sequence produced in the same study (AY854644, Bourhy et al, 2005), as well as the Charleville virus sequence found

in our study. Given that our sequence is supposedly from the same strain (MRM4059) as that used by Bourhy et al, and that Almpiwar virus has been found to be serologically distinct from Charleville virus, (Tesh et al, 1983, Calisher et al, 1989), one plausible hypothesis is that the Almpiwar virus sequence from Bourhy et al (2005) is a Charleville virus contaminant and not Almpiwar virus.

Our phylogenetic analysis found strong support for the inclusion of all known rhabdoviruses of squamate reptiles except Marco virus in a clade that has previously been termed the Almpiwar group (Bourhy et al, 2005). Our analysis, as well as those of Bourhy et al (2005) and Dacheux et al (2010), finds that Humpty Doo virus is also well supported as a member of this clade. Humpty Doo virus has been isolated from two different hematophagous insect species, *Culicoides marksii* and *Forcipomyia (Lasiohelix)* sp., which are biting midges (Standfast et al, 1984). Vertebrate hosts for Humpty Doo have not yet been identified; we hypothesize that a squamate host is likely.

All reported isolations of Almpiwar virus and Charleville virus from vertebrate hosts have been from squamates. Immunodiagnostically, one of 99 humans in Australia had a positive serum neutralization test for Charleville virus (Doherty et al, 1973). One of 133 humans in Australia, 4 of 75 *C. virgatus* skinks, 3 of 91 cattle, 2 of 55 horses, 1 of 36 sheep, 1 of 53 kangaroos, 1 of 39 bandicoots, and 1 of 35 wild birds had positive serum neutralization tests for Almpiwar virus; however, the authors expressed a lack of confidence in the significance of these results (Doherty et al, 1970). A number of rhabdoviral phylogenetic analyses have found that relatedness may differ from serologic results (Bourhy et al, 2005; Dacheux et al, 2010; Gubala et al, 2010). Our findings are similar, further disagreeing with the lack of serologic findings of relatedness of Almpiwar/Charleville viruses with Chaco/Timbo viruses, and the serologic cross-reactivity of Charleville virus with the lyssaviruses (Calisher et al, 1989).

Marco, Chaco, and Timbo viruses were not isolated from any of 2,095 humans or 20,344 other vertebrates (Causey et al, 1966). However, Marco, Chaco, and Timbo viruses were also not isolated from any of 68,758 pools of arthropods, even though experimental infections showed that Marco and Chaco viruses multiplied in *Aedes aegypti* salivary glands (Causey et al, 1966). The presence of the caiman lizard rhabdovirus in numerous erythrocytes implies that very high titers may be present, and this may be why these viruses were successfully isolated from lizards, but not from arthropods, where they are also likely to be present.

Oak Vale virus, isolated from two Australian mosquito species, *Culex edwardsi* and *Ochlerotatus vigilax* (Gubala, 2009), has been found in one previous analysis to be in the Almpiwar group (Bourhy et al, 2005). However, a later analysis found that inclusion of a number of additional viruses resulted in the inclusion of Oak Vale virus in a new sister group to the Almpiwar group, termed the Sandjimba group (Dacheux et al, 2010). No phylogenetic analyses including the Sandjimba group have found monophyly of Oak Vale virus and the Almpiwar group (Quan et al, 2011). All known vertebrate hosts of the Sandjimba group are birds (Dacheux et al, 2010).

Marco virus clusters within a Dimarhabdovirus clade called the Hart Park group (Bourhy et al, 2005; Gubala et al, 2010). Viruses in this clade have unusually large genomes with several additional genes that are not yet well understood (Gubala et al, 2008; Gubala et al, 2010). The Hart Park group contains viruses utilizing diverse vertebrate hosts, including Ngaingan virus found in Australian macropods and cattle (Gubala et al, 2010), Flanders virus found in North American passerine birds (Whitney, 1964), and our results show the use of squamate hosts. The ability of these viruses to use vertebrate hosts from diverse

classes is notable. Baby mice inoculated intracranially with Marco virus have been found to have shorter survival times than those infected with Chaco, Timbo, or Almpiwar virus (Causey et al, 1966; Monath et al, 1979). Unlike Chaco, Timbo, or Almpiwar virus, Marco virus grew to significant titers in Vero cells at 37 °C, and was the only one of the four to produce CPE in *Terrapene* heart (TH-1) cells (Monath et al, 1979).

Medicine has traditionally waited for viruses to cause epidemics or epizootics before significant surveillance occurs. With our increased understanding of virus ecology and evolution, it becomes more feasible to identify probable candidates for future novel disease outbreaks, and increase surveillance. Due to lack of proofreading by their polymerases and constant selective pressure by the host immune system, RNA viruses have the fastest mutating genomes found in nature, and tracking evolution of these viruses is highly clinically significant. Chandipura virus has only recently emerged as a cause of large outbreaks of human encephalitis (Rao et al, 2004). Emerging disease is frequently associated with host switches. One meta-analysis of human diseases found that 816 of 1407 (58%) are zoonotic, and of human diseases, zoonotic diseases are significantly more likely to be emerging (Woolhouse and Gowtage-Sequeria, 2005). The ability of the Hart Park group to utilize diverse hosts indicates that further understanding and surveillance of this virus group would be prudent.

Although the South American squamate rhabdoviruses have all been isolated from members of the family Teiidae, they do not represent the largest group of animals that have been sampled. There were 2447 specimens of *Tropidurus torquatus* (family Iguanidae) examined, representing 51% of the 4766 lizards surveyed by Causey et al (1966), and the Iguanidae represented 59.7% of sampled lizards, but no rhabdoviruses were isolated from lizards in this family. In contrast, Causey et al grew 14 rhabdovirus isolates from the 1855 teiid lizards (38.9% of lizards sampled). The small numbers of skinks (38, 0.8% of lizards sampled) and geckos (1, 0.02% of lizards sampled) prevent drawing conclusions about comparative prevalence, but Almpiwar and Charleville viruses have been isolated from Australian skinks and geckos, respectively, indicating rhabdoviruses can infect these clades. The teiids, skinks and geckos all represent more basal divergences within the squamates. The iguanids, snakes, varanids, chameleons, agamids, and anguids form a large monophyletic clade known as the Toxicofera that includes nearly 60% of squamate species (Vidal and Hedges, 2005). The failure to isolate rhabdoviruses from this clade is notable.

In agreement with Sasaya et al (2002), the distance found in our analysis between the polymerase of Lettuce Big vein virus, a non-Rhabdoviral *Varicosavirus*, and many of the rhabdoviruses was less than that between the rhabdoviral genus *Novirhabdovirus* and other rhabdoviruses. Varicosaviruses are double-stranded segmented RNA viruses and are not enveloped, unlike rhabdoviruses. This implies that these traits may not always be segregating, or that the polymerase gene history may differ in this case from that of the rest of the virus. Although recombination is generally considered uncommon in *Mononegavirales*, it does occur (Chare et al, 2003).

Further understanding of the diversity of the *Rhabdoviridae* is needed. Many areas of the phylogenetic analysis are not well resolved. Spring viraemia of carp virus and *Scophthalmus maximus* rhabdovirus are now recognized as members of the genus *Vesiculovirus*. Our analysis finds them separated only by one node with poor support values (53% posterior probability, <50% ML bootstrap) and very short branch lengths, which is certainly not evidence for paraphyly but does not identify the monophyly. The small genome size of rhabdoviruses places limitations on phylogenetic resolution, and the best way to improve this is through including further taxa in analyses. The availability of a more complete representation of existing species for comparison results in greater phylogenetic resolution

(Flynn et al., 2005, Stefanovic et al., 2004). Significant errors can occur in phylogenetic analyses due to incomplete taxon sampling, even if very large sequence length is assessed (Lunter, 2007). However, further sequence data of these viruses will also likely improve resolution and is also needed. While much of the genomes of rhabdoviruses cannot be reliably aligned, knowledge of genomic structure is important for an understanding of rhabdovirus virology. This is especially of interest for Marco, since the Hart Park group have complex genome structures with several additional genes (Gubala et al, 2008).

In conclusion, we find that most known rhabdoviruses of squamates cluster in the Almpiwar subgroup. The exception is Marco virus, which clusters with the Hart Park group that have diverse vertebrate hosts. We report that a caiman lizard rhabdovirus forms inclusions in erythrocytes, which presents a reasonable route for infecting hematophagous insects. Further studies are needed to determine the ecology and clinical significance of these viruses.

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Figure 1. Erythrocytes of a caiman lizard (*Dracaena guianensis*) with intracytoplasmic rhabdoviral inclusions marked by arrows. The scale bar on the bottom right is 5 microns.

Figure 2. Electron micrograph of intracytoplasmic inclusions in caiman lizard erythrocytes, showing particles morphologically consistent with rhabdoviruses. Scale bar = 200 nm.

Figure 3.

Bayesian phylogenetic tree of predicted 106–145 amino acid sequences of RNA-dependent-RNA polymerase sequences based on MAFFT alignment. Multifurcations are marked with arcs. Bayesian posterior probabilities of clusters as percentages are in bold, and ML bootstrap values for clusters based on 350 re-samplings are given to the right. Nyamanini virus, a non-rhabdovirus member of the *Mononegavirales* used as an outgroup. Lettuce big-vein associated virus, a member of the unassigned non-*Mononegavirales* genus *Varicosavirus*, was included. The squamate rhabdoviruses sequenced in this study are indicated with arrows. Brackets demarcate viral clades. Accession numbers of sequences retrieved from GenBank are given after the name.