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Source: Journal of Zoo and Wildlife Medicine, 48(2):420-430.
Published By: American Association of Zoo Veterinarians
https://doi.org/10.1638/2014-0200R4.1
URL: http://www.bioone.org/doi/full/10.1638/2014-0200R4.1

Journal of Zoo and Wildlife Medicine 48(2): 420–430, 2017

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Abstract: Ferlaviruses (FV, previously referred to as ophidian paramyxoviruses, OPMV), are enveloped viruses with a negative-strand RNA genome, affecting snakes in captivity worldwide. Infection is characterized by respiratory and nervous clinical signs and carries high mortality rates, but no specific treatment or vaccine is currently available. Costa Rica has 16 species of vipers, found in captivity in collections essential for antivenom production, reintroduction, and public education. FV circulation in these populations was previously unknown, and the risk of introducing the viruses into naïve collections or free-ranging populations exists if the virus's presence is confirmed. The objective of this study was to determine seroprevalence and FV shedding in 150 samples from captive vipers in nine collections across Costa Rica. A hemagglutination inhibition (HI) assay was performed to determine the antibody titer against two *Ferlavirus* strains, Bush viper virus (BV) and Neotropical virus (NT), and reverse-transcriptase polymerase chain reaction (RT-PCR) and sequencing to determine virus secretion in cloacal swabs. *Ferlavirus* strains were replicated in Vero cells, and chicken anti-FV polyclonal antibodies were produced and used as a positive control serum for the HI. Results demonstrate that seroprevalence of anti-FV antibodies in viper serum was 26.6% ($n = 40$) for the BV strain and 30% ($n = 45$) for the NT strain in the population tested. Furthermore, molecular characterization of FV group A was possible by sequencing the virus recovered from three cloacal swabs, demonstrating circulation of FV in one collection. This study demonstrates for the first time serological evidence of FV exposure and infection in vipers in captivity in Costa Rica, and suggests cross reactivity between antibodies against both strains. Appropriate biosafety measures could prevent the spread of FV between and within collections of reptiles in the country.

Key words: Captivity, *Ferlavirus*, hemagglutinin-inhibition assay, titers, vipers.

INTRODUCTION

Ferlaviruses (FV, previously referred to as ophidian paramyxoviruses, OPMV) are enveloped viruses with a 15.3-kb negative-stranded RNA genome, belonging to the *Ferlavirus* genus, family Paramyxoviridae.[2,9] The *Ferlavirus* strain Fer-de-Lance paramyxovirus (type species) was first diagnosed as the causative agent of acute deaths in fer-de-lance vipers (*Bothrops moojeni*) in captivity in Switzerland in 1972,[5] and was later identified as the cause of death of approximately 60% of vipers in a collection in the United States.[10]

FV infect the Elapidae, Boidae, Colubridae, Pythonidae, and Viperidae families in captivity,[2,18] causing an acute, chronic, or subclinical presentation with unspecific signs of respiratory and nervous disease.[13] The virus can be transmitted between snakes by direct contact with infected nasal, oral, and/or cloacal discharges.[21]

No vaccine against FV is currently available;[11] thus, a combination of serological and virus detection tests are essential to assess exposure, circulation, and shedding of the viruses in collections and to establish biosafety and quarantine controls.[21] Exposure to FV and subsequent serological conversion can be assessed by detecting specific antibodies against the viruses with the use of the hemagglutinin-inhibition (HI) assay.[10] Although this test has been broadly used to determine anti-FV antibodies, some challenges have been identified, such as low reproducibility between laboratories and false-negative results due to lack of cross reactivity between different viral strains.[3] *Ferlavirus* infection and shedding can be assessed by detecting the RNA viral genome by polymerase chain reaction (PCR),[2] and isolation of the viruses in cell culture.[16]

Sixteen species of vipers are distributed in the wild in Costa Rica and are found in public and private collections throughout the country; these collections are essential for antivenom production, breeding, reintroduction, and public education.[25] The prevalence of antibodies against FV in snakes in captivity in Costa Rica is unknown, and

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the risk of introducing the viruses into naïve collections or free-ranging populations exists if the virus's presence is confirmed.[4] The objectives of this study were to determine the prevalence of antibody against two *Ferlavirus* strains in vipers in captivity in Costa Rica, and to determine shedding of the virus and molecularly characterize the strain circulating in the animals sampled. Findings will aid to understand the antibody response and cross-reaction against *Ferlavirus* strains in snakes in captivity. Furthermore, it will identify if *Ferlavirus* strains are circulating and will assess if biosafety and quarantine measures need to be enhanced in collections across the country.

MATERIALS AND METHODS

Sample collection

Sampling protocols were approved by the National Commission for the Biodiversity Management of Costa Rica (CONAGEBIO, San Jose, Costa Rica; R-011[bis]-2010-OT-CONAGEBIO, R-013-2010-OT-CONAGEBIO, and R-012-2010-OT-CONAGEBIO).

One hundred fifty blood samples were collected from snakes (Viperidae family) in nine different collections in Costa Rica between 2009 and 2011. Collections were located in the provinces of San Jose ($n = 5$), Cartago ($n = 1$), Alajuela ($n = 1$), and Heredia ($n = 2$), and were labeled from A to I (Fig. 1; Table 1). Blood samples (0.5 to 1 ml) were collected from the ventral coccygeal vein and the serum was separated, aliquoted, and stored at −20°C until further processing.

To determine if FV were being shed, a cloacal swab was obtained from each snake. A new, sterile swab was inserted into the cloaca, gently rotated two to three times, and placed in a new plastic tube containing 1 ml of DMEM (Sigma-Aldrich, St. Louis, Missouri, 63103, United States; D1145), supplemented with 5% fetal bovine serum (Sigma-Aldrich; F6178) and 1% antibiotic and antimycotic (10,000 units penicillin, 10 mg streptomycin and 25 µg amphotericin B per milliliter; Sigma-Aldrich; A5955). Samples were vortexed and frozen at −70°C until further processing.

Cell-culture replication of Bush viper and Neotropical *Ferlavirus* strains

Two commercially available *Ferlavirus* strains belonging to two different genogroups, Bush viper (BV, group B) (American Type Culture Collection, Manassas, Virginia 20110, USA; ATCC VR-1408) and Neotropical (NT, group A) (ATCC VR-1409), were cultured and used in the HI assay.[24] Each strain was passaged three times in Vero cells (African Green monkey epithelial kidney cells) grown at 28°C in DMEM (Sigma-Aldrich; D1145) supplemented with 5% fetal bovine serum (Sigma-Aldrich; F6178) and 1% antibiotic and antimycotic (10,000 units penicillin, 10 mg streptomycin and 25 µg amphotericin B per milliliter; Sigma-Aldrich; A5955).[16] Cells and supernatant were collected after 5 days and samples were freeze-thawed once and clarified by centrifugation at 400 *g* for 5 min. The supernatant was collected and the amount of hemagglutinin-neuraminidase protein present was tested with the use of a hemagglutination test (HA),[13,16] and the presence of viral RNA was confirmed by conventional RT-PCR and sequencing (see methods below).[2] The viruses were aliquoted and stored at −70°C until further processing.

FV replication in embryonated chicken eggs

Nine-day-old embryonated Cobb chicken eggs (National Service for Animal Health, SENASA, 3-3006 Cenada, Heredia, Costa Rica) were inoculated in the allantoic sac with *Ferlavirus* strains as described previously;[1] two eggs were inoculated with the BV strain, two with the NT strain, and two with uninfected media to serve as negative controls. The eggs were incubated at 28°C for 6 days. Subsequently, the allantoic fluid was collected and tested for hemagglutination activity with the use of the HA,[13] and the presence of viral RNA was confirmed by conventional RT-PCR and sequencing.[2]

Hemagglutination test (HA)

The hemagglutinating activity of the viral culture and the allantoic fluid from infected eggs was assessed. With the use of V-bottom, 96-well plates (Greiner Bio One, Monroe, North Carolina 28110, United States; 651101), twofold serial dilutions of 100 µl of each strain was performed in phosphate-buffered saline solution (PBS, GE Healthcare, HyClone Laboratories, Legan, Utah 84321, United States; SH3025601), and 50 µl of a solution of 1% chicken erythrocytes was added. The plate was incubated at 4°C for 30 min and the hemagglutinating units/ml of the viruses were determined by calculating the reciprocal of the highest dilution of virus solution that resulted in complete agglutination of the erythrocytes.[13]

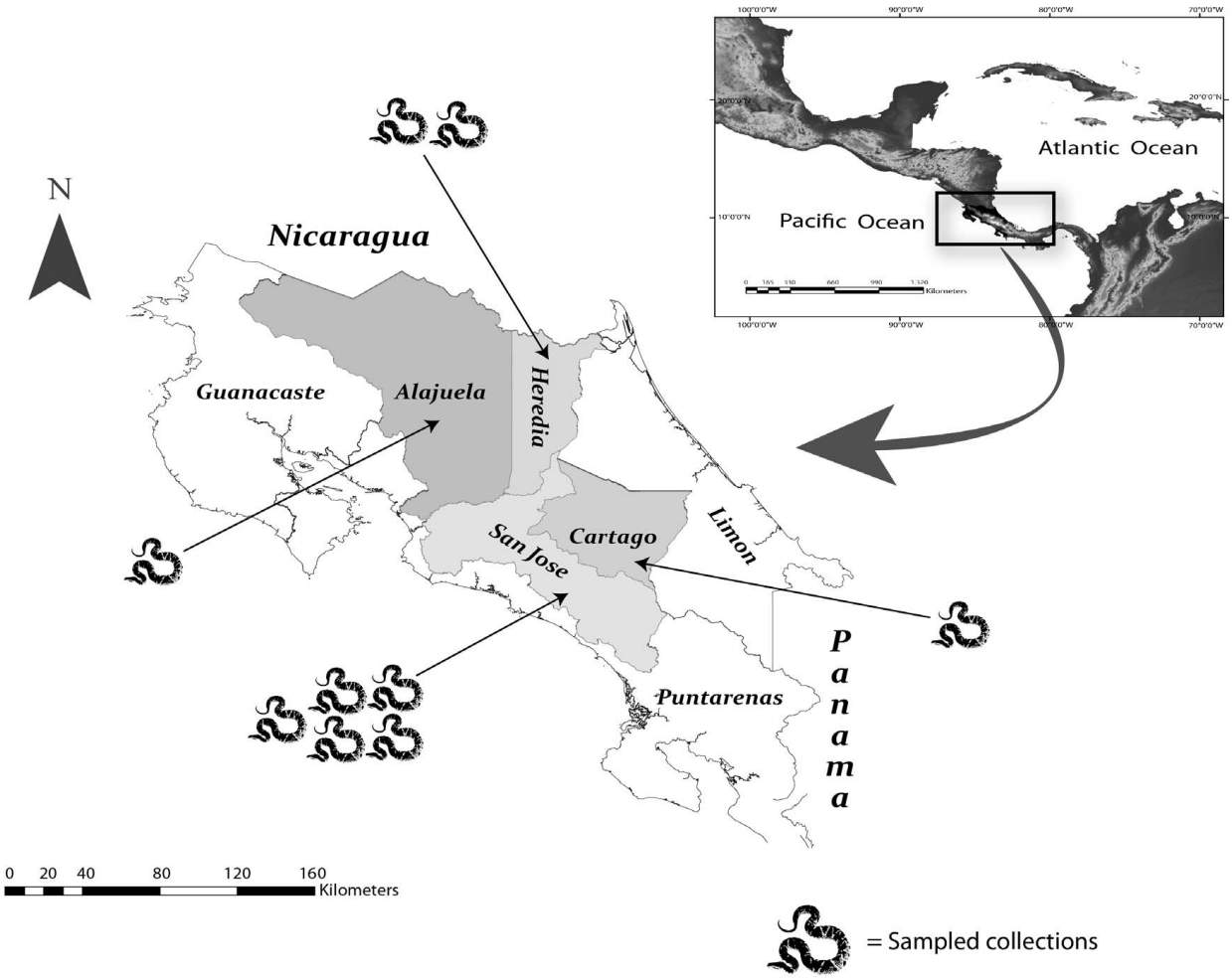

Figure 1. Number of collections visited per province of Costa Rica; each snake represents one collection. The geographic region of Central America is represented in the upper right corner, and the specific provinces of Costa Rica are described in the map; grey-scale provinces represent sampled areas. Bar scale represents kilometers (Arcgis V.10, Adobe Illustrator CS5).

FVs polyclonal antibody production

Animal handling protocol and sampling procedures were approved by the Committee of Animal Welfare of the Universidad Nacional de Costa Rica (EMV-CBA-010-2010).

To produce polyclonal antibodies against the viruses, six 4-wk-old Cobb chickens, derived from a commercial farm and serologically negative for FV, were immunized with the BV ($n = 3$) or NT strain ($n = 3$); three chickens were immunized with media and served as negative controls.[8] For the initial immunization, two different emulsions were prepared with 1 ml of the previously described FV-infected allantoic fluids (containing 640 HA units/ml for BV and 160 HA units/ml for NT) and an equal volume of Freund's Complete Adjuvant (FCA, Sigma-Aldrich, F5881; 0.25 ml/chicken). Each chicken received 0.5 ml of the suspension injected intramuscularly (IM).

A booster was administered 2 weeks later, inoculating 0.5 ml of the infected allantoic fluid IM without the FCA, and 0.5 ml of the supernatant of the third cell culture passage of each strain intravenously (IV; containing 320 HA units/ml for BV and 80 HA units/ml for NT). Thirty-five days after the first immunization, blood was collected from the chickens and serum was separated and tested to assess the antibody activity by means of the HI assay.[10]

FVs HI assay

This assay was performed as described previously.[10] Briefly, serum was diluted 1 : 5 in PBS, heated to 56°C for 30 min, and absorbed with pelleted chicken erythrocytes at 4°C for 12 hr. Subsequently, twofold serial dilutions of the sera were carried out in columns through a V-bottom, 96-well plate (Greiner Bio One; 651101) for a final

Table 1. Number and percentage (in parentheses) of samples classified by genus, species, and collection; common names of vipers are shown below

		Genus and species sampled *n* (%)											
		Crotalus			*Bothrops*	*Bothriechis*		*Atropoides*		*Cerrophidion*	*Lachesis*	*Porthidium*	
Collection	Province	*C. simus*[a]	*C. vegrandis*[b]	*C. adamanteus*[c]	*B. asper*[d]	*B. lateralis*[e]	*B. schlegelii*[f]	*A. mexicanus*[g]	*A. picadoi*[h]	*C. godmani*[i]	*L. stenophrys*[j]	*P. nasutum*[k]	Total
A	San Jose	42	–	–	23	–	–	–	–	–	–	–	65 (43.3)
B	San Jose	2	–	–	3	–	–	–	–	–	–	–	5 (3.3)
C	San Jose	1	–	–	–	4	4	–	–	2	–	–	11 (7.3)
D	San Jose	5	–	–	–	2	–	–	2	2	–	–	11 (7.3)
E	San Jose	5	–	–	2	2	–	–	1	–	–	1	11 (7.3)
F	Heredia	–	–	–	–	2	4	–	1	–	3	–	10 (6.7)
G	Heredia	–	–	2	6	1	–	1	3	–	–	–	13 (8.7)
H	Cartago	–	–	–	–	4	4	–	–	–	–	–	8 (5.4)
I	Alajuela	4	3	3	4	–	–	1	–	–	1	–	16 (10.7)
Total		59 (39.3)	3 (2.0)	5 (3.3)	38 (25.3)	15 (10)	12 (8.0)	2 (1.3)	7 (4.7)	4 (2.7)	4 (2.7)	1 (0.7)	150 (100)

[a] Central American rattlesnake.
[b] Uracoan rattlesnake.
[c] Eastern diamondback rattlesnake.
[d] Fer-de-lance.
[e] Side-striped palm viper.
[f] Eyelash pit viper.
[g] Central American jumping pit viper.
[h] Jumping pit viper.
[i] Godoman's pit viper.
[j] Central American bushmaster.
[k] Rainforest hog-nosed pit viper.

volume of 25 µL of the diluted sample. Each *Ferlavirus* strain was diluted to contain 8 HA units/0.025 ml, and 25 µl of BV or NT was added to the serum and incubated for 1 hr at room temperature; all sera were tested against each virus. After incubation, 25 µl of a solution of 1% chicken erythrocytes was added and incubated at 4°C for 30 min. The reading of HI was performed visually after the incubation period, and the titer was calculated as the reciprocal value of the last dilution that inhibited hemagglutination. Control sera were included on each plate with the use of the chicken anti-BV and anti-NT polyclonal serum as positive control, and the serum from the mock-infected chickens as negative control. The HA activity of both diluted strains was verified on each plate.

A sample was considered positive if the last dilution where the inhibition of hemagglutination observed was greater than 1 : 10 (3.3 $\log_2$). The positive samples were then classified as low (>1 : 10–80; >3.3–6.3 $\log_2$), moderate (>1 : 80–160; >6.3–7.3 $\log_2$), and high (>1 : 160; >7.3 $\log_2$) positives. A sample was considered negative if it contained a titer equal to or lower than 1 : 10 (≤3.3 $\log_2$).[3]

RNA extraction, RT-PCR, and electrophoresis

Viral RNA was extracted from 250 µl of the supernatant of the viral cultures, allantoic fluid recovered from infection eggs, and the media of the cloacal swabs of each sampled snake. RNA was extracted using TRIzol® (Thermo Fisher Scientific, Inc., Waltham, Massachusetts, 2451, USA; 10296-010) following the manufacturer's instructions. The RNA was re-suspended with 50 µl of deionized, diethylpyrocarbonate (DEPC) -treated water (Thermo Fisher Scientific; RO601), and the sample was converted to cDNA immediately using the Revert Aid™ kit (Fisher Scientific; K1632) following the manufacturer's instructions.

The nested RT-PCR was performed as described previously to amplify a part of the large RNA-dependent RNA polymerase (L) gene.[2] In the first round of amplification, 25 µl of PCR Master Mix (Thermo Fisher Scientific; K0171), 1 µl of forward primer 5F (5′- GCAGAGATTTTCTCTTTCTT -3′), 1 µl of reverse primer 6R (5′- AGCTCT-CATTTTGTATGTCAT -3′, both in a concentration of 5 µM), and 21 µl of nuclease-free water (Thermo Fisher Scientific; R0581) were mixed with 2 µl of cDNA.[2] The amplification program was as follows: 95°C for 2 min; 30 cycles of 95°C for 30 sec, 40°C for 30 sec, and 72°C for 1 min; and held at 4°C.[2]

The second round of amplification was performed with 25 µl of PCR Master Mix (Thermo Fisher Scientific; K0171), 1 µl of 5 µM forward primer 7F (5′- TAGAGGCTGTTACTGCTGC -3′), 1 µl of 5 µM reverse primer 8R (5′- CATCTTGGCAAATAATCTGCC -3′), and 21 µl of nuclease-free water (Thermo Fisher Scientific; R0581) mixed with 2 µl of the product of the first amplification.[2] The amplification program was the same as described for the first round.[2]

The PCR products of the second round of amplification were separated in a 2% agarose gel with the use of Gel Red (Biotium, Hayward, California, 94545, USA; 41003) staining and UV transillumination, and the expected product was 566 base pairs (bp) long.[2]

Sequencing and phylogenetic analysis

PCR products of positive samples were run in a 2% agarose gel a second time; bands were cut and purified with the use of the PureLink® Quick Gel Extraction Kit (Invitrogen, Löhne, D-32584, Germany; K2100-12). The quality of the purified products was verified on a 1% agarose gel, and DNA concentration was read using the spectrophotometer Nanodrop 2000 (Thermo Scientific).

The sequencing reaction was performed with the use of the Big Dye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Austin, Texas, 78744, USA; 4337455). Briefly, 4 µl of the ready reaction premix was mixed with 2 µl of the Big Dye® sequencing buffer, 3.2 pmol of primer, and 25 ng of DNA, for a final volume of 20 µl. The sequencing reaction program was performed as recommended by the manufacturer. The products were purified by gel filtration chromatography with the use of the PureLink® quick PCR purification kit (Invitrogen; K3100-01) and read on the Genetic Analyzer 3130 (Applied Biosystems, Thermo Fisher Scientific) in the Centro de Investigaciones en Biologia Celular y Molecular of the Universidad de Costa Rica.

Sequences were edited and processed using the program Geneious v.6.1.8 (Biomatters, Auckland, 1010, New Zealand), and compared to public access sequences in GenBank (National Center for Biotechnology Information [NCBI], Bethesda, Maryland 20894, USA) with the use of the Basic Local Alignment Search Tool (BLAST). Alignment of sequences was performed with the ClustalW algorithm of Geneious program.[7] Fer-de-Lance paramyxovirus L gene sequence (GenBank Accession No. NC_005084) was used as the reference strain and Sendai virus L gene sequence (NC_001552) as outgroup in the phylogenetic

analysis. Genetic distances were determined with the use of the Tamura-Nei genetic distance model and the neighbor-joining tree build method; the bootstrap resampling method was applied.[7]

Statistical analysis

For the HI assay, each sample was tested twice on each plate; the final antibody titer was $\log_2$-transformed for normalization, and the final titer was reported as the geometric mean of both titers. The percentage of positive animals to FV antibodies was calculated per genera of snake.

Pearsons's correlation test was performed to determine the association between the HI antibody titers against both strains with the use of SPSS software version 17.0 (IBM, White Plains, New York 10604, USA); a P value ≤ 0.05 was considered significant.

RESULTS

Sample collection

Blood samples were obtained from snakes belonging to nine different collections located in four provinces of the country (Fig. 1). Snakes were chosen randomly depending on the availability of the species in each collection; at least 25% of the total viper population in each collection was sampled (excluding Collection I, where less than 10% of vipers were sampled due to permit requirements).

The total of samples, genus, and species for each collection sampled are described in Table 1. Animals from 11 species, out of seven genera, were sampled: *Crotalus* was the most prevalent ($n = 67$; 44.6%) (Table 1).

Cell culture replication of BV and NT *Ferlavirus* strains

Vero cells grown at 28°C were used to passage the BV and NT strains. Evidence of viral replication of both strains in cell culture was observed after the fifth day postinoculation as cytopathic effects consisting of round cells and syncytial and cellular lysis.

The HA for BV after the third passage was 320 HA units/ml, and the highest HA for NT was obtained after the second passage with 640 HA units/ml.

FVs replication in embryonated chicken eggs

Four 9-day-old embryonated chicken eggs were used to replicate the *Ferlavirus* strains for 6 days at 28°C. The effect of the viruses resulted in embryos of smaller size with skin hemorrhage in all virus-inoculated eggs; this was not observed in the controls.

The allantoic fluid was collected and tested for hemagglutination activity. The HA activity for the eggs infected with BV was 640 HA units/ml and for NT was 160 HA units/ml. The controls were negative for HA activity.

FV polyclonal antibody production

To obtain polyclonal antibodies against the viruses and use them as positive controls for the HI assay, groups of three 4-wk-old Cobb chickens were immunized with the BV or NT strain; three chickens served as negative controls. Sera from all chickens were negative for FV antibodies in a pre-immune HI test performed on Day 0, and the immunization of the chickens resulted in HI antibody titers as high as 1 : 640 for the BV strain, and 1 : 1280 for the NT strain when sampled 35 days after the first inoculation. Sera from the controls were negative for FV antibodies throughout the experiment.

FV HI

An HI assay was used to assess the antibody titers against strains BV and NT in sera from 150 vipers. Based on the results of the HI assay, the total seroprevalence of anti-FV antibodies was 26.6% ($n = 40/150$) against the BV strain and 30.0% ($n = 45/150$) against the NT strain (Table 2). The positive samples were further subdivided into low, moderate, and high titers depending on the HI result (Table 2).

Positive samples belonged to snakes of seven collections located in three different provinces of the country (Table 3). The highest proportion of positive samples belonged to collection A, with 46.1% of samples positive against BV and 47.6% positive against NT; samples belonging to collections E and I were negative in the HI.

Crotalus was the genus that presented the highest number of positive samples against both strains ($n = 33/67$ against BV and 34/67 against NT, Fig. 2, Table 3). For the samples positive against the BV strain, snakes belonging to the genus *Atropoides* presented the highest titer mean (8.8 ± 3.5 SD $\log_2$), followed by *Crotalus* (8.0 ± 1.4 SD $\log_2$), *Bothrops* (7.3 $\log_2$), and *Bothriechis* (6.1 ± 3.4 SD $\log_2$). The samples belonging to the genera *Lachesis*, *Cerrophidium*, and *Porthidium* were all negative for antibodies against this strain (Fig. 2).

Table 2. Prevalence of antibodies against *Ferlavirus* Bush viper (BV) and Neotropical (NT) strains in serum samples and frequencies of the negative and positive results (low, moderate, and high).

Hemagglutination inhibition categories	Prevalence anti-BV in samples *n* (%)	Prevalence anti-NT in samples *n* (%)
Positive (>1 : 10; >3.3 $\log_2$)	40 (26.6)	45 (30.0)
Low (>1 : 10–80, >3.3–6.3 $\log_2$)	17 (11.3)	22 (14.6)
Moderate (>1 : 80–160; ≥6.3–7.3 $\log_2$)	12 (8.0)	6 (4.0)
High (>1 : 160; >7.3 $\log_2$)	11 (7.3)	17 (11.4)
Negative (≤1 : 10; 3.3 $\log_2$)	110 (73.4)	105 (70.0)
Total	150 (100)	150 (100)

For the samples positive against the NT strain, *Atropoides* presented a higher titer mean (8.8 ± 3.5 SD $\log_2$), followed by *Crotalus* (7.4 ± 1.9 SD $\log_2$), *Bothrops* (7.3 $\log_2$), and *Bothriechis* (4.9 ± 2.0 SD $\log_2$). The samples belonging to the genera *Lachesis*, *Cerrophidium*, and *Porthidium* were negative for antibodies against this strain (Fig. 2).

A moderate, significant correlation was observed between antibody titers against BV and NT strains ($r^2 = 0.73$, $P < 0.0001$; Fig. 3). The majority of positive snakes had antibodies against both strains; a difference in titers was only observed in four animals of the genus *Bothriechis* belonging to two collections, and one animal of the genus *Crotalus*, which had low-positive titers against NT but were negative for antibodies against BV.

RNA extraction, nested RT-PCR, electrophoresis, and sequence analysis

The RT-PCR assay was performed to test for the presence of FV-L gene RNA in cell culture, allantoic fluid recovered from the infected eggs, and cloacal swabs of all the snakes sampled. The expected 566-bp amplicon was observed in all the supernatants of cell culture and allantoic fluid after RT-PCR.

Only three of the cloacal swabs were positive in the nested RT-PCR; they belonged to snakes of the genus *Bothriechis* from collection H, presented no clinical signs of disease, and were negative for FV antibodies.

All positive swab samples and the cell culture supernatant were analyzed to construct a phylogenetic tree; samples are shown in bold (Fig. 4). The cell culture supernatant (BV3P-ATCC and NT3P-ATCC) clustered inside the respective group of FV. The three cloacal samples (Both-CR 1, 2, and 3) clustered together with a 99.8% identity between each other, and they revealed a 99% identity with the FV-group A sequence Dasy-GER00 (Fig. 4).

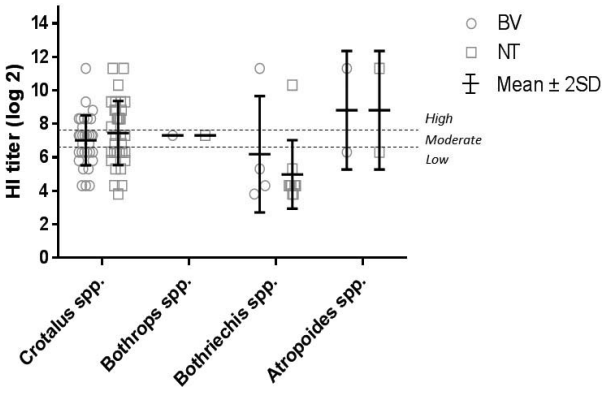

Figure 2. Hemagglutination inhibition titer ($\log_2$) of positive samples against *Ferlavirus* Bush viper (BV) and Neotropical (NT) strains, per genera. Open symbols represent blood samples from individual vipers; mean ± 2 standard deviations (SD) are shown as black lines. Positive samples were further classified as low (>3.3–6.3 $\log_2$), moderate (>6.3–7.3 $\log_2$), or high (>7.3 $\log_2$), and are separated by dashed lines.

DISCUSSION

This study investigated the antibody response and cross reaction against *Ferlavirus* strains in

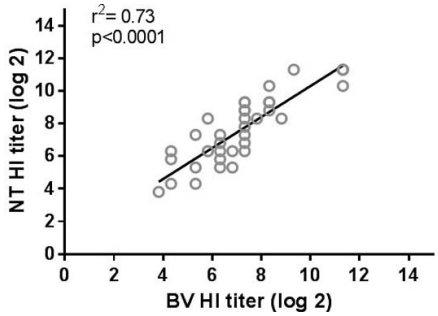

Figure 3. Correlation between HI titers ($\log_2$) of positive animals against *Ferlavirus* Bush viper (BV) and Neotropical (NT) strains. Open symbols represent blood samples from individual vipers. A linear regression was calculated and is represented as a line in the graph.

Table 3. Prevalence of positive samples against *Ferlavirus* Bush viper (BV) and Neotropical (NT) strains per genera and collection. The total number of samples is represented as number of positive samples/number total samples tested (percentage of positive samples).

		Crotalus		*Bothrops*		*Bothriechis*		*Atropoides*		*Cerrophidion*	*Lachesis*	*Porthidium*	Total	Total
Collection	Province	BV	NT	BV	NT	BV	NT	BV	NT	BV/NT	BV/NT	BV/NT	BV	NT
A	San Jose	29/42	30/42	1/23	1/23	–	–	–	–	–	–	–	30/65 (46.1)	31/65 (47.6)
B	San Jose	1/2	1/2	0/3	0/3	–	–	–	–	–	–	–	1/5 (20)	1/5 (20)
C	San Jose	0/1	0/1	–	–	1/8	4/8	–	–	0/2	–	–	1/11 (9)	4/11 (36.6)
D	San Jose	1/5	1/5	–	–	0/2	0/2	2/2	2/2	0/2	–	–	3/11 (27.2)	3/11 (27.2)
E	San Jose	0/5	0/5	0/2	0/2	0/2	0/2	0/1	0/1	–	–	0/1	0/11	0/11
F	Heredia	–	–	–	–	1/6	2/6	0/1	0/1	–	0/3	–	1/10 (10)	2/10 (20)
G	Heredia	2/2	2/2	0/6	0/6	0/1	0/1	0/4	0/4	–	–	–	2/13 (15.3)	2/13 (15.3)
H	Cartago	–	–	–	–	2 /8	2/8	–	–	–	–	–	2/8 (25)	2/8 (25)
I	Alajuela	0/10	0/10	0/4	0/4	–	–	0/1	0/1	–	0/1	–	0/16	0/16
Total		33/67 (49.2)	34/67 (50.7)	1/38 (2.6)	1/38 (2.6)	4/27 (14.8)	8/27 (29.6)	2/9 (22.2)	2/9 (22.2)	0/4	0/4	0/1	40/150 (26.6)	45/150 (30.0)

snakes in captivity in Costa Rica, and identified a *Ferlavirus* strain circulating in a collection, stressing the necessity for biosafety and quarantine controls in collections across the country.

FV seroprevalence were 26.6% for antibodies against BV, and 30% against NT; the majority of HI-positive samples were classified as low positives, with titers ranging from >10 to 80 (>3.3–6.3 $\log_2$). Previously, a study demonstrated that serological response against FV does not remain elevated for long periods of time, but decreases a few months after the initial infection.[10] Considering that only three snakes tested positive for FV-RNA in the swabs, and had no detectable FV antibodies in serum, possibly due to an acute infection, the low titer of antibodies measured in the remaining snakes could indicate a possible clearance of the virus.[9,14] To assure that these collections are truly negative for virus circulation, antigen testing should be performed periodically.[21]

Animals belonging to the *Crotalus* genus presented the highest number of positive samples in the HI assay per genera for both strains; however, the majority of positive snakes belonged to the same collection, and these results could be due to a sampling bias. Because of the differences in number of animals and genera sampled per collection in this study, it was not possible to perform a statistical analysis to determine the effect of snake genus or collection on FV prevalence. Nevertheless, it has been suggested that animals belonging to the *Crotalus* genus present a greater susceptibility to infection by FV,[10] although this still needs to be demonstrated in a controlled experiment.

The HI assay is an easy, practical, and low-cost method to diagnose exposure of animals to FV.[10] In this study, *Ferlavirus* strains BV and NT were passaged three times in Vero cells and once in embryonated chicken eggs, and used to perform the HI assay and to induce chicken anti-FV polyclonal antibodies to be used as controls for the assay.[1,16] High titers of antibodies against BV and NT strains were generated, demonstrating the suitability of chickens to produce anti-FV polyclonal antibodies. Rabbits were used previously as source of antibodies against BV and NT strains, although the HI titers produced were not reported.[24] Chickens are easy animals to work with, produce large amounts of antibodies in response to foreign antigens, and did not present any clinical sign of disease; thus, should be considered for anti-FV antibody production.[8]

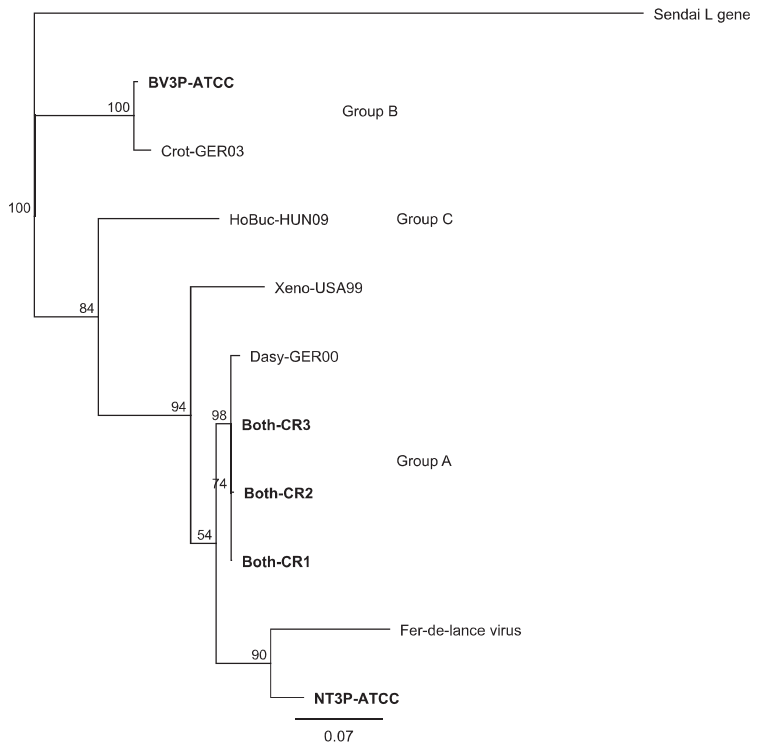

Figure 4. Phylogenetic distance tree using partial gene sequences of the large RNA-dependent RNA polymerase (L) gene of reptilian Ferlaviruses, nt 566 bp. Genetic distances were determined using the Tamura-Nei genetic distance model and the neighbor-joining tree build method; bootstrap values are indicated beside the branches.[7] Sendai virus (GenBank Accession No. NC_001552) was used as outgroup and Fer-de-Lance paramyxovirus (NC_005084) as the reference strain. Sequences in bold represent the samples recovered from cell culture (BV3P-ATCC, NT3P-ATCC) and cloacal swabs (Both-CR 1, 2, and 3) in this study. Xeno-USA99 / Dasy-GER00 (GQ277613), CrotGER03 (G0277611), and HoBuc-HUN09 (JX186195) represent reptile sequences belonging to FV groups A, B and C, respectively.

A weakness of the HI assay is the inability to distinguish the strain to which the animal was exposed due to cross reactivity.[3,6] In the current study, the positive correlation observed between antibody titers towards BV and NT could be interpreted as a close antigenic relationship between strains.[12] Cross reactivity of BV and NT strains against a rabbit polyclonal antibody has been demonstrated previously,[24] and it has now been assessed with the use of snake sera. More studies are needed to establish the genetic background and pathogenicity of these *Ferlavirus* strains.[20]

Snakes belonging to seven out of the nine collections sampled were positive to FV antibodies, demonstrating previous exposure to FV. In order to reduce the introduction of the virus into collections, a quarantine period of at least 90 days and disinfection of cages could reduce the cases of FV infection.[23,26] Furthermore, it is recommended that an animal has a negative RT-PCR test for shedding before introduction.[21]

RT-PCR is a quick and sensitive assay that can be used to determine an active virus infection and shedding directly.[15] *Ferlavirus* shedding can be detected in choana or cloacal swabs, and fluid recovered from tracheal washes, using RT-PCR or virus isolation in cell culture.[22] In the present study, a cloacal swab was taken from each snake and stored at −70°C in cell culture media; only three animals resulted positive to the test. An insufficient amount of RNA in the samples[17] or viral clearance by the animals could have caused the low number of positive samples observed in this study.[22]

The type of sample collected and the time after infection should also be considered for RNA testing. A recent experimental infection study demonstrated that FV-RNA is detectable 28 days postinfection in cloacal swabs, compared to 16 days post-infection using tracheal wash fluid.[22] In the present study, a snake with neurological signs, belonging to the same collection where the viral strain was recovered, was FV RT-PCR positive in

a tissue homogenate (results not shown), but negative in the RT-PCR on the cloacal swab performed ante mortem. This further demonstrates the importance of the type of sample to be collected for FV detection and should be considered for future studies.[22]

Phylogenetic analysis of FV of reptiles has clustered strains into at least three genogroups: A, B, and the newly described C.[19] There is limited sequence variance between groups and strains do not cluster based on the reptile species affected.[15] In this study, three animals from the genus *Bothriechis* tested positive for shedding of FV. Phylogenetic analysis performed on viral RNA demonstrated that the strain that circulated in this collection belongs to group A, clustering close to the snake strain Dasy-GER00 and NT. Even though little is known about the differences in clinical manifestations and virulence between *Ferlavirus* strains, a recent experimental study demonstrated that a group B strain caused greater pathological lesions, compared to group A and C strains.[22] Seropositivity of the snakes in the current study could be due to the phylogenetic relationship between the circulating strain and NT. As only one *Ferlavirus* strain was recovered from the cohort, this statement cannot be confirmed until more sequences are available.

In summary, this study demonstrates serological evidence of viral exposure to FV in four genera of vipers, and the circulation of an FV-group A strain in one of the collections in Costa Rica. More studies are needed to determine the antibody kinetics in the animals and to assess the risk of introduction of this virus into native free-ranging populations in the country.

Acknowledgments: The authors would like to acknowledge the Universidad Nacional de Costa Rica for financial support, owners and keepers of the collections for their collaboration, Mrs. Rocio Cortes for her aid in laboratory procedures, Mr. Jose Hernandez from the National Commission for the Biodiversity Management of Costa Rica for his aid in the government permits, Mr. Reinaldo Pereira of Universidad de Costa Rica, and Markus Czub, Regula Waeckerlin, Juan Carlos Corredor and Narges Nourozieh of the University of Calgary for the aid in analysis and writing of this paper, and the anonymous reviewers for their useful comments, which significantly improved this manuscript.

LITERATURE CITED

1. Ahne W, Adrian J, Mayr A. Replication of reptilian paramyxovirus in avian host systems. Zentralbl Veterinarmed B. 1999;46(1):57–62.

2. Ahne W, Batts WN, Kurath G, Winton JR. Comparative sequence analyses of sixteen reptilian paramyxoviruses. Virus Res. 1999;63(1–2):65–74.

3. Allender MC, Mitchell MA, Dreslik MJ, Phillips CA, Beasley VR. Measuring agreement and discord among hemagglutination inhibition assays against different ophidian paramyxovirus strains in the Eastern massasauga (*Sistrurus catenatus catenatus*). J Zoo Wildl Med. 2008;39(3):358–361.

4. Allender MC, Mitchell MA, Phillips CA, Gruszynski K, Beasley VR. Hematology, plasma biochemistry, and antibodies to select viruses in wild-caught Eastern massasauga rattlesnakes (*Sistrurus catenatus catenatus*) from Illinois. J Wildl Dis. 2006;42(1):107–114.

5. Clark HF, Lief FS, Lunger PD, Waters D, Leloup P, Foelsch DW, Wyler RW. Fer de Lance virus (FDLV): a probable paramyxovirus isolated from a reptile. J Gen Virol. 1979;44(2):405–418.

6. Franke J, Essbauer S, Ahne W, Blahak S. Identification and molecular characterization of 18 paramyxoviruses isolated from snakes. Virus Res. 2001; 80(1–2):67–74.

7. Hall BG. Phylogenetic trees made easy. 4th ed. Sunderland (MA): Sinauer Associates; 2011. p. 69–90.

8. Hodek P, Stiborová M. Chicken antibodies—superior alternative for conventional immunoglobulins. Proc Indian Nat Sci Acad. 2003;B69(4):461–468.

9. Hyndman TH, Shilton CM, Marschang RE. Paramyxoviruses in reptiles: a review. Vet Microbiol. 2013;165(3–4):200–213.

10. Jacobson ER, Gaskin J, Wells S, Bowler K, Shumacher J. Epizootic of ophidian paramyxovirus in a zoological collection: pathological, microbiological, and serological findings. J Zoo Wildl Med. 1992; 23(3):318–327.

11. Jacobson ER, Gaskin JM, Flanagan JP, Odum RA. Antibody responses of western diamondback rattlesnakes (*Crotalus atrox*) to inactivated ophidian paramyxovirus vaccines. J Zoo Wildl Med. 1991;22(2): 184–190.

12. Kindermann J, Kübber-Heiss A, Kerschbaumer P, Nowotny N. Phylogenetic analysis of the L and HN gene of ophidian paramyxoviruses. Brief report. Arch Virol. 2001;146(5):1021–1035.

13. Kolesnikovas CK, Grego KF, Rameh de Albuquerque LC, Jacobson ER, Monezi TA, Mehnert DU, Catão-Dias JL. Ophidian paramyxovirus in Brazilian vipers (*Bothrops alternatus*). Vet Rec. 2006;159(12):390–392.

14. Lloyd C, Manvell R, Drury S, Sainsburry AW. Seroprevalence and significance of paramyxovirus titres in a zoological collection of lizards. Vet Rec. 2005;156(18):578–580.

15. Marschang RE, Papp T, Frost JW. Comparison of paramyxovirus isolates from snakes, lizards and a tortoise. Virus Res. 2009;144(1–2):272–279.

16. Mayr A, Franke J, Ahne W. Adaptation of reptilian paramyxovirus to mammalian cells (Vero cells). J Vet Med B Infect Dis Vet Public Health. 2000;47(2):95–98.

17. Mraz M, Malinova K, Mayer J, Pospisilova S. MicroRNA isolation and stability in stored RNA samples. Biochem Biophys Res Commun. 2009; 390(1):1–4.

18. Orós J, Sicilia J, Torrent A, Castro P, Déniz S, Arencibia A, Jacobson ER, Homer BL. Immunohistochemical detection of ophidian paramyxovirus in snakes in the Canary Islands. Vet Rec. 2001;149(1):21–23.

19. Papp T, Gál J, Abbas MD, Marschang RE, Farkas SL. A novel type of paramyxovirus found in Hungary in a masked water snake (*Homalopsis buccata*) with pneumonia supports the suggested new taxonomy within the *Ferlavirus* genus. Vet Microbiol. 2013;162(1): 195–200.

20. Papp T, Pees M, Schmidt V, Marschang RE. RT-PCR diagnosis followed by sequence characterization of paramyxoviruses in clinical samples from snakes reveals concurrent infections within populations and/or individuals. Vet Microbiol. 2010;144(3–4):466–472.

21. Pasmans F, Blahak S, Martel A, Pantchev N. Introducing reptiles into a captive collection: the role of the veterinarian. Vet J. 2008;175(1):53–68.

22. Pees M, Neul A, Muller K, Schmidt V, Truyen U, Leinecker N, Marschang R. Virus distribution and detection in corn snakes (*Pantherophis guttatus*) after experimental infection with three different ferlavirus strains. Vet Microbiol. 2016;182(1):213–222.

23. Pees M, Schmidt V, Marschang RE, Heckers, Krautwald-Junghanns ME. Prevalence of viral infections in captive collections of boid snakes in Germany. Vet Rec. 2010;166(14):422–425.

24. Richter GA, Homer BL, Moyer SA, Williams DS, Scherba G, Tucker SJ, Hall BJ, Pedersen JC, Jacobson ER. Characterization of paramyxoviruses isolated from three snakes. Virus Res. 1996;43(1):77–83.

25. Solorzano A. Serpientes de Costa Rica; 2004. Santo Domingo de Heredio (Costa Rica): Editorial INBio. p. 612–721.

26. Woodford MH. Quarantine and health screening protocols for wildlife prior to translocation and release into the wild. Paris (France): IUCN Species Survival Commission's Veterinary Specialist Group, Office International des Epizooties (OIE), Care for the Wild, and the European Association of Zoo and Wildlife Veterinarians [Internet] 2000 [cited 2016 May 16]: p. 64. Available from: http://www.cwhcrcsf.ca/wildlife_health_topics/risk_analysis/Quarantine.pdf

Accepted for publication 4 January 2017