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Preliminary design and evaluation of an RT-PCR assay for detecting *Reptarenavirus* in snakes (subfamily *Boinae* and families *Pythonidae* and *Colubridae*) from the Reserva Natural Bioparque Wakatá, Colombia

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ABSTRACT

Inclusion body disease (IBD) is a viral infection of snakes caused by *Reptarenavirus*, characterized primarily by neurological signs, although it can also be subclinical. This study preliminarily designed and evaluated an RT-PCR assay to detect the virus in snakes from the Wakatá Biopark (Colombia), using two sets of primers and without a positive control. Although the results were negative and the assay proved unviable for routine use, the study generated valuable information as a basis for future projects addressing this problem and provided recommendations for the differential diagnosis of the disease.

Keywords (MeSH): reverse transcriptase polymerase chain reaction, snakes, *Reptarenavirus*, wild animals.

Diseño y evaluación preliminar de una prueba de RT-PCR para la detección de *Reptarenavirus* en serpientes (subfamilia *Boinae* y familias *Pythonidae* y *Colubridae*) de la Reserva Natural Bioparque Wakatá, Colombia

RESUMEN

La enfermedad de cuerpos de inclusión (ECI) es una afección viral de serpientes causada por *Reptarenavirus*, con signos clínicos principalmente neurológicos, aunque puede ser subclínica. Este estudio diseñó y evaluó, de forma preliminar, una prueba RT-PCR en un intento de detectar el virus en serpientes del Bioparque Wakatá (Colombia), utilizando dos *sets* de *primers* y sin un control positivo. Aunque los resultados fueron negativos y la prueba no mostró ser viable para uso rutinario, se generó información valiosa como

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base para futuros proyectos acerca de este problema y se hacen recomendaciones acerca del diagnóstico diferencial de la enfermedad.

Palabras clave (DeCS): Reacción en cadena de la polimerasa de transcriptasa inversa, serpientes , *Reptarenavirus*, animales silvestres .

Introduction

Inclusion body disease (IBD) is a transmissible viral disorder of captive and wild snakes, caused by infection with viruses of the genus *Reptarenavirus* (Alfaro-Alarcón *et al.*, 2022). IBD primarily affects boas of the subfamily *Boinae* and pythons of the family *Pythonidae* (Thiele *et al.*, 2023). The clinical manifestations are predominantly neurological and include loss of righting reflex, opisthotonos (commonly referred to as "star-gazing" or "corkscrew posture"), head tremors, flaccid paralysis, disorientation, and regurgitation (Argenta *et al.*, 2020). However, in many cases the disease remains subclinical, with affected individuals serving as asymptomatic carriers for extended periods and presenting nonspecific signs such as progressive emaciation, secondary infections, and sudden death (Ossiboff, 2018; Simard *et al.*, 2020).

Transmission of the disease has been reported to occur both horizontally and vertically. In horizontal transmission, snakes shed viral RNA through the skin, feces, and urates (Dervas, 2024), whereas vertical transmission may occur from either parent (Alfaro-Alarcón *et al.*, 2022; Dervas, 2024). A potential vector under consideration is the cosmopolitan snake mite *Ophionyssus natricis*, commonly known as the reptile mite (Mendoza-Roldan *et al.*, 2023). In addition to causing irritation, anemia, and even death in severe infestations, this ectoparasite has been proposed as a possible vector of IBD, although conclusive evidence remains lacking (Chang & Jacobson, 2010; Dervas, 2024).

From a geographical perspective, IBD is considered a globally relevant disease, with cases reported worldwide. Several studies have documented its occurrence in snakes under human care in North America, Europe, Asia, and Australia. Currently, no cure is available, and euthanasia of infected animals is commonly practiced to protect the health of the remaining population (Alfaro-Alarcón *et al.*, 2022).

Regarding differential diagnoses, other viruses are also capable of producing inclusion bodies and may cause diseases with clinical signs similar to those of IBD, including *Herpesvirus*, *Adenovirus*, and *Paramyxovirus* (Marschang, 2014). Among these, *Paramyxoviruses* are the most relevant, as they also form intracytoplasmic inclusion bodies and produce neurological signs comparable to those observed in IBD.

According to the International Committee on Taxonomy of Viruses (ICTV), five species of *Reptarenavirus* are currently recognized: *R. giessenae*, *R. commune*, *R. californiae*, *R. aurei*, and *R. rotterdamense*. However, several additional unclassified species remain under provisional and non-official designations (Radoshitzky *et al.*, 2023). The *Reptarenavirus* genome consists of a bisegmented single-stranded RNA with an ambisense coding strategy (De la Torre, 2020; Hetzel *et al.*, 2021). The first segment, designated S (short), encodes the glycoprotein precursor and the nucleoprotein (NP), while the second

segment, designated L (long), encodes the zinc-finger matrix protein (ZP) and the RNA-dependent RNA polymerase (RdRp) (Lintala *et al.*, 2022b; Radoshitzky *et al.*, 2023).

Several authors have demonstrated that snakes affected by IBD frequently exhibit coinfections with multiple *Reptarenavirus* species, as evidenced by the presence of more than one type of S and L segment in a single individual (Argenta *et al.*, 2020). Moreover, higher levels of the S segment have been correlated with a greater abundance of inclusion bodies (Thiele *et al.*, 2023). These findings are consistent with previous studies characterizing the composition of inclusion bodies, which revealed that they are primarily composed of NP encoded by the S segment (Chang *et al.*, 2013; Hetzel *et al.*, 2021; Lintala *et al.*, 2022a; Thiele *et al.*, 2023).

In general, animals affected by IBD develop inclusion bodies (IBs) in most tissues. Consequently, the historical antemortem diagnostic method of choice has been the detection of IBs in samples such as blood smears and, most commonly, liver biopsies (Alfaro-Alarcón *et al.*, 2022). However, although IB detection is a rapid and cost-effective diagnostic tool, it is not fully reliable, as IB formation appears to vary both among individuals and across species (Hetzel *et al.*, 2021; Thiele *et al.*, 2023).

In a significant proportion of cases, snakes may develop chronic forms of the disease in which, despite harboring characteristic intracytoplasmic IBs, they exhibit no obvious clinical signs. Conversely, some individuals have been documented with clinical manifestations consistent with IBD but lacking detectable IBs (Alfaro-Alarcón *et al.*, 2022; Argenta *et al.*, 2020; Dervas, 2024; Lintala *et al.*, 2022b; Thiele *et al.*, 2023).

Other methods for detecting *Reptarenavirus* include immunohistochemistry targeting the NP protein, transmission electron microscopy for direct viral visualization, next-generation sequencing (NGS), Sanger sequencing, viral inoculation in cell cultures, ELISA, and Western blotting (Alfaro-Alarcón *et al.*, 2022; Chang *et al.*, 2013; Hetzel *et al.*, 2013; Ihász *et al.*, 2022; O'Rourke & Lertpiriyapong, 2015). Nevertheless, these approaches are not yet suitable for routine diagnosis, either because of their high cost or the limited accessibility of the required technologies.

Due to the genetic variability of the virus and the limited information available on the disease in Latin America, diagnosing and reporting its presence remain challenging, as the specific *Reptarenavirus* species and/or variants circulating within snake populations in Colombia are still unknown. According to records from the Animal Health Coordination of the RNBW, an ongoing issue has been observed since 2019, in which several snakes, including both boas and pythons, have exhibited clinical signs and postmortem lesions consistent with IBD (figure 1). In some of these cases, characteristic IBs of IBD have also been detected in blood and tissue samples (figure 1).

Given the suspicion of IBD, several individuals were euthanized due to compromised health status and as an epidemiological preventive measure against potential transmission. In light of these circumstances, the aim of this study was to design and preliminarily evaluate an RT-PCR assay to detect *Reptarenavirus* in snakes under professional care at the RNBW.

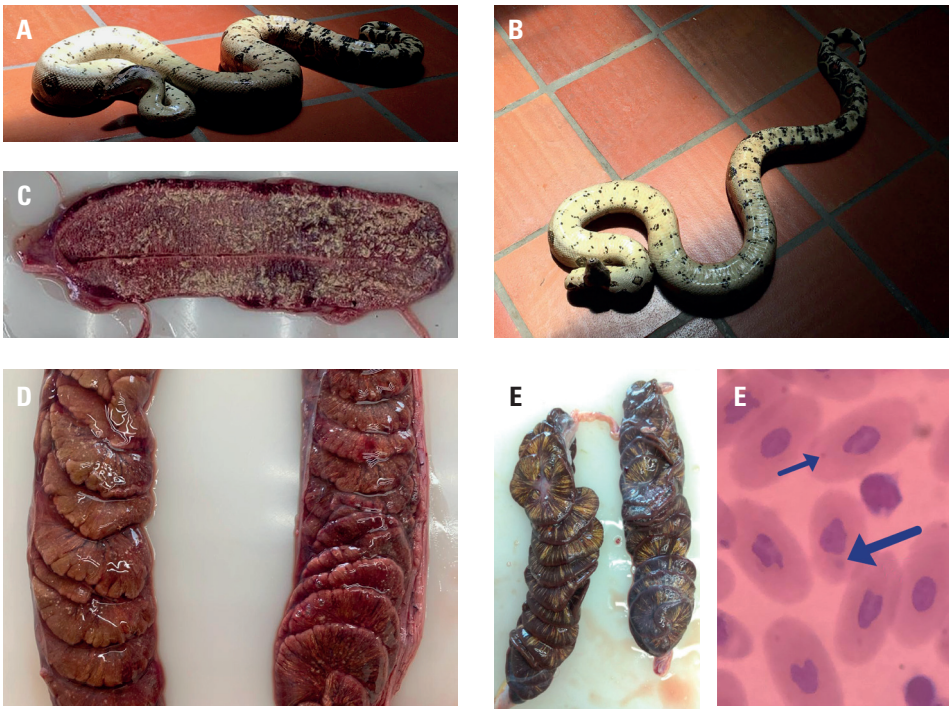

FIGURE 1. (A) Boa (*Boa constrictor*) exhibiting "star-gazing" posture. (B) Boa (*Boa constrictor*) showing delayed or absent righting reflex. (C) Lung of a boa (*Boa constrictor*) with pyogranulomatous material compatible with bacterial pneumonia, with IBs reported in the histopathological report. (D) Kidneys of a boa (*Boa constrictor*) showing multiple whitish pinpoint foci within the renal parenchyma, with IBs confirmed in the histopathological report. (E) Kidneys of another boa (*Boa constrictor*) with renal calcification and IBs identified in the histopathological report. (F) Hematoxylin and eosin–stained blood smear from one of the boas at the RNBW, showing several amphophilic intracytoplasmic IBs characteristic of IBD (arrows).
Source: RNBW, 2018-2020.

Materials and methods

Ethics committee

This study was approved by the Research, Ethics, and Bioethics Committee of the Fundación Parque Jaime Duque, as well as by the corresponding Subcommittee of Research, Ethics, and Bioethics of the RNBW, in accordance with the Research Policy of the Fundación Parque Jaime Duque (Approval Act No. 005, Project Code 0016, approved on December 6, 2023). In addition, it was approved by the Committee for the Care and Use of Animals in Research (CICUA) of Universidad de La Salle under reference FUA No. 102-2023 (version 03), approved on April 1, 2024.

Study design and location

The study was conducted at the RNBW facilities located within the Fundación Parque Jaime Duque, in the municipality of

Tocancipá, Cundinamarca, approximately 20 km north of Bogotá. Molecular analyses were performed at the José Joaquín Vargas Muñoz Laboratory of Universidad de La Salle and at the Institute of Genetics of the National University of Colombia, both in Bogotá.

Population and samples

The study population consisted of 15 snakes. Sampling was conducted opportunistically and based on convenience, prioritizing individuals that exhibited the greatest number of historical and/or current clinical signs consistent with IBD. This approach was selected because the remaining snakes appeared clinically healthy but were considered suspect cases due to their proximity and potential exposure. Sample collection was carried out between February and June 2024.

The population comprised individuals born at the biopark as well as snakes obtained through seizures and donations. However, the exact geographic origins and lineages of these individuals remain unknown. It is assumed that all snakes originated within the national territory, with the exception of non-native species (Pythonidae).

Blood samples were collected by venipuncture of the ventral coccygeal vein under physical restraint, following all RNBW protocols and parameters for handling, animal welfare, and sample collection established for the species (Divers & Stahl, 2019). A total of 14 individuals (93%) were successfully sampled. The distribution of individuals is presented in table 1.

It is noteworthy that one of the brown boas, BCH1 (*Epicrates maurus*), had to be euthanized, and tissue samples were collected from the liver, brain, heart, muscle, and from abnormal cysts observed in the musculature and skin of the cranial third of the body. No samples could be obtained from one *Boa constrictor* individual (BC6), which cohabited with two conspecifics that were successfully sampled; therefore, the results from the latter are theoretically extrapolatable to the former.

RT-PCR Design

Primer design

We sought a tool to computationally optimize primer selection targeting a conserved region shared among multiple

TABLE 1. Snakes sampled in the study and designation assigned to each individual

Species	Common name	Number of individuals	Study designation
Boa constrictor	Common boa	6	BC1, BC2, BC3, BC4, BC5 y BC6
Eunectes murinus	Green anaconda	1	A
Lampropeltis micropholis	Ecuadorian milksnake	1	FC
Epicrates maurus	Brown rainbow boa	2	BCH1 y BCH2
Python molurus	Indian python	3	P1, P2 y P3
Python curtus	Sumatran short-tailed python	1	PS
Python molurus bivittatus	Burmese python	1	PA

Source: own elaboration.

Reptarenavirus sequences. For this purpose, the ConsensusPrime tool was used (Collatz *et al.*, 2022). As input, we used *Reptarenavirus* S-segment sequences reported in GenBank. The tool was run according to the authors' instructions under default parameters (Collatz *et al.*, 2022). The candidate primers are listed in table 2.

RNA extraction

Blood samples were processed with various reagents for RNA extraction using the Invi-Sorb® Spin Universal Kit. All manufacturer's instructions for viral RNA extraction from liquid samples were followed, with the only modification being an extension of the incubation time of the lysis buffer, RNA carrier, proteinase K, and blood sample mixture from the recommended 10 minutes to 30 minutes. This adjustment was implemented to ensure more efficient lysis and RNA recovery. For tissue samples, all manufacturer's instructions for viral RNA extraction from biopsy tissues were strictly followed.

cDNA synthesis

Complementary DNA (cDNA) was synthesized from the RNA obtained in the previous step using random hexamer primers and the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific), following the manufacturer's protocol.

cDNA PCR

The cDNA product was subsequently subjected to PCR amplification using

TABLE 2. Primers used in this study, showing 100% identity and cases with 1-2 mismatches according to BLAST. UGV: University of Giessen Virus; ROUTV: Boa Arenavirus NL; RNI: unidentified *Reptarenavirus*

Primer	Sequence	Amplicon size (bp)	100% ID	1–2 mismatches
NP1F	ACATTGGATCAACTCCTCAT	252	• UGV-1	
			• UGV-2	
			• UGV-3	• RNI
NP1R	ATGTTGTCACCCTTTCAAAG		• ROUTV	
			• RNI	
NP2F	ATGTGTCCTGAGGAATTGAT	229	• UGV-1	
			• UGV-2	
			• UGV-3	• RNI
NP2R	GACCAAACAACCCAACATTA		• UGV-4	
			• RNI	

Source: own elaboration.

DreamTaq DNA Polymerase (Thermo Fisher Scientific). A temperature gradient was initially performed to determine the optimal annealing temperature, testing 50.0, 50.7, 52.0, 53.9, 56.3, 58.3, 59.4, and 60.0 °C. All reactions were carried out in a BIO-RAD® C1000 thermal cycler. The initial master mix formulation is shown in Table 3. Throughout the study, specific parameters of the master mix and PCR conditions were modified to optimize amplification, including increasing magnesium concentration, adjusting the amount of cDNA template, and testing different annealing temperatures.

Visualization of PCR products

For the visualization of results, BIO-RAD® Mini-Sub-Cell GT electrophoresis chambers were used. Initially, a 1% agarose gel was prepared and run at 100 V for 60 minutes. Subsequently, the agarose concentration was increased to 2% and the electrophoresis time extended to 75 minutes. The sizes of the amplified products visualized in the gel were then compared with the expected theoretical sizes for each primer set. For this purpose, a molecular size marker with fragments of 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1300, and 2500 base pairs (bp) was employed. Additionally, Red Gel stain, which intercalates into DNA and fluoresces under UV light, was used together with a UV transilluminator to visualize the amplified products.

DNA purification and sanger sequencing

The DNA obtained from the amplification of cDNA from selected samples was purified for subsequent Sanger sequencing to confirm the identity of the bands observed in agarose gels. Duplicate master mixes with a final volume of 50 µl, including 6 µl of template, were prepared. The bands visualized in the gels were excised and purified using the QIAquick® Spin Kit (QIAGEN), following the manufacturer's instructions for DNA extraction from gels.

Ten microliters of each purified product were submitted without prior confirmation of DNA concentration (required: 10 pg/µl). However, based on the intensity of the bands observed in the purification

TABLE 3. Initial master mix used for cDNA amplification. The volume of each component and its final concentration are shown

Component	Volume (µl)	Final concentration
Forward and reverse primers	1.25 µl	0.5 pmol
dNTPs	0.5 µl	0.2 mmol
10× Buffer	2.5 µl	1x
DNase-free water	18.25 µl	NA
DreamTaq DNA Polymerase	0.25 µl	0.05 U/µl
cDNA template	1 µl	NA
Total	25 µl	

Source: own elaboration.

confirmation gel, it was assumed that the concentration was adequate. In addition, 5 µl of both primers from the NP1 set, at a concentration of 5 pmol/µl, were also submitted.

In a second round, purified products obtained using the same methodology were analyzed at the Universidad Nacional de Colombia. DNA concentrations were measured with a Nanodrop Lite Plus spectrophotometer (Thermo Fisher Scientific), yielding average values of 10 ng/µl, which were considered optimal for sequencing. Consequently, 10 µl of each purified sample, together with 5 µl of primer NP1F at 5 pmol/µl, were submitted to the Institute of Genetics of the same university for sequencing.

Results and discussion

Two *Boa constrictor* individuals (BC1 and BC2) were initially selected empirically as positive controls, as they exhibited the highest number of clinical signs consistent with the disease. Both presented respiratory symptoms, flaccid paralysis, tremors, ataxia, partial loss of righting reflex, and severe immunosuppression, all associated with IBD. However, the initial PCR results under temperature gradient conditions did not show detectable bands in the agarose gel, which may suggest either a low concentration of viral RNA or a suboptimal reaction.

For this reason, PCR parameters were systematically adjusted to determine the combination that yielded the most reliable amplification. Magnesium concentration, template cDNA volume, and annealing temperature were evaluated. The optimal conditions were determined to be a magnesium concentration of 2.75 mmol, 2 µl of cDNA template, and an annealing temperature of 55.0 °C or 53.9 °C (figure 2).

Additionally, to confirm the absence of cross-reactivity between primers, a negative control (C–) consisting of a reaction without template cDNA was included (figure 2). In parallel, a non-target DNA control of Avian Infectious Laryngotracheitis (CILT) was incorporated to rule out nucleic acid degradation or the presence of reaction inhibitors (figure 2). Regarding primer performance, NP1 primers demonstrated the highest efficiency. This was evidenced by the appearance of amplicon bands matching the theoretical size of the expected products, whereas NP2 primers did not yield consistent bands (figure 2). Using NP1 primers, two recurrent bands were observed across all snake samples belonging to the subfamily Boinae: one of approximately 220–250 bp, corresponding to the theoretical size expected for *Reptarenavirus* amplification with NP1 (252 bp), and another of ~500 bp (figure 2). Based on these findings, the 220–250 bp bands were suspected to indicate the presence of *Reptarenavirus*, which prompted the extension of sampling to the remaining individuals in the population.

Due to the suspected presence of the virus based on the bands observed in snakes of the subfamily *Boinae*, the 220–250 bp and ~500 bp bands were purified for Sanger sequencing. Blood from *Boa constrictor* BC2 and a liver sample obtained at necropsy from the chocolate boa BCH1 were used. The chromatograms obtained showed mixed, low-intensity signals, suggesting either contamination or the presence of multiple DNA sequences within the same band. Ultimately, BLAST analysis did not yield significant alignments with existing databases.

Subsequently, to improve band separation and rule out the possibility of co-migrating amplicons of similar size,

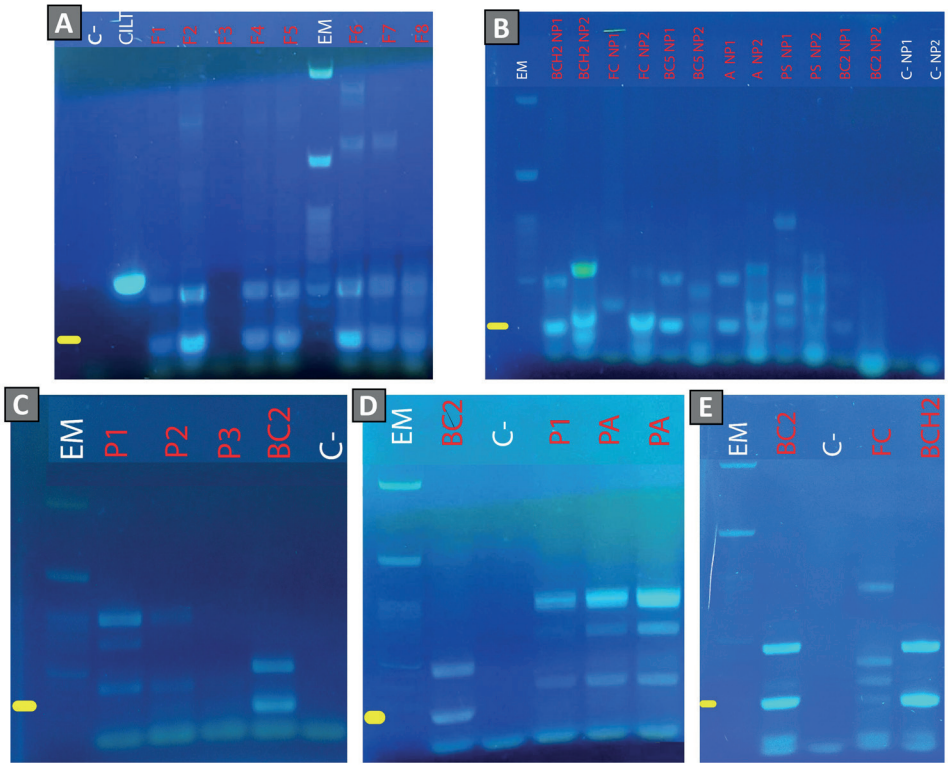

FIGURE 2. Agarose gels of PCR-amplified cDNA. In all gels, a molecular weight marker (EM) is shown. The yellow dash indicates the theoretical size of 250 bp expected for the presence of *Reptarenavirus.* (A) PCR results of samples from the experimental group designated F, performed with 2.75 mM $MgCl_2$ and an annealing temperature of 53.9 °C. This group included samples obtained from the necropsy of the chocolate boa (BCH1) and blood from Boa constrictor individuals (BC), labeled as follows: 1–Heart, 2–Liver, 3–Cyst, 4–Muscle, 5–Brain, 6–Blood BC2, 7–Blood BC3, 8–Blood BC4. A negative control (C–) and a non-target cDNA control of Avian Infectious Laryngotracheitis (CILT) were also included. (B) PCR results of blood samples from the chocolate boa BCH2, Boa constrictor BC5 and BC2, anaconda (A), Sumatran python (PS), and false coral snake (FC). In this experimental group, both NP1 and NP2 primers were tested, each with their respective negative controls (C–). (C, D, E) PCR results of samples from Indian pythons P1, P2, and P3, Boa constrictor BC2, chocolate boa BCH2, albino python (PA), and false coral snake (FC), all amplified with NP1 primers using 2.75 mM $MgCl_2$ and an annealing temperature of 53.9 °C.

Source: own elaboration.

electrophoresis parameters were optimized by using 2% agarose, which provided better resolution of the observed bands. At this stage, dimethyl sulfoxide (DMSO) was also tested, as it is known to enhance PCR specificity and stability. The addition of 1 µl increased band intensity without altering the reactions. Based on these results, new PCR assays were performed with blood samples from BC2, BCH2, and A1, as well as liver tissue from BCH1, under the following conditions: addition

of DMSO, 3 mM magnesium, annealing temperature of 55 °C, 2% agarose, and electrophoresis at 100 V for 75 min. Bands of 220–250 bp and ~500 bp were again detected, along with additional bands, with similarities noted among boas and differences compared to pythons (figure 3). The ~500 bp band proved difficult to purify (figure 3), restricting extraction to the 220–250 bp bands and a 300 bp band from A1. Following gel confirmation (figure 3), these products were submitted for Sanger sequencing using NP1F primers. The sequencing results produced chromatograms of higher purity and stronger signal compared to those obtained previously (figure 4).

Based on the sequencing results, the 220–250 bp bands obtained from all samples were analyzed. Visual inspection of the chromatograms indicated high similarity among these fragments, and subsequent analysis confirmed that they shared more than 92% identity. This suggests that the 220–250 bp bands represent the same DNA sequence across different individuals. However, BLAST analysis did not reveal significant alignments with available databases. In contrast, the 300 bp sequence obtained from the anaconda showed partial alignment with messenger RNA from Boinae species (70–88% identity). These findings led us to hypothesize that the consistently observed 220–250

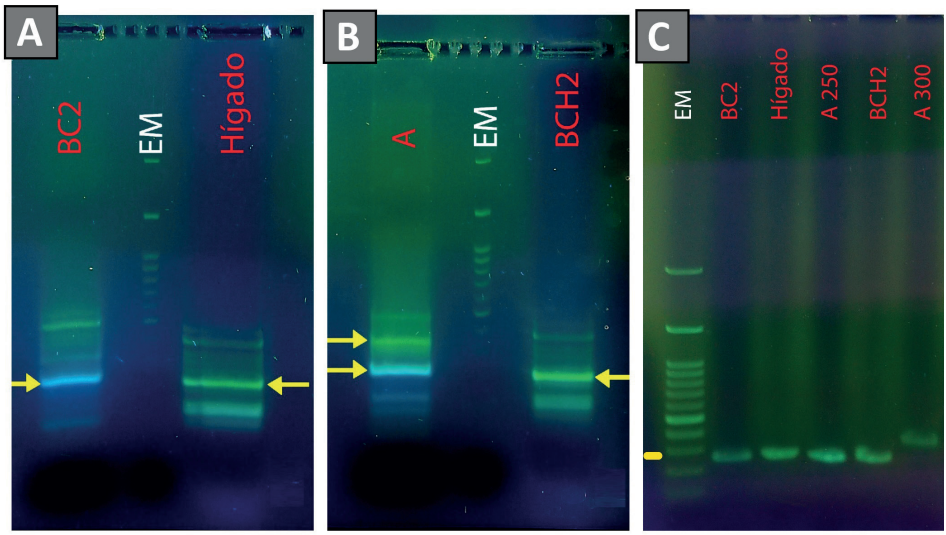

FIGURE 3. A molecular ladder (EM) is included in all gels. The yellow marker indicates the theoretical size of 250 bp, corresponding to the expected amplicon of *Reptarenavirus*. (A, B) Agarose gels showing extraction and purification of cDNA, with the purified bands marked by arrows. PCR amplicons were obtained from cDNA of blood samples from Boa constrictor BC2, anaconda A, chocolate boa BCH2, and liver tissue from the necropsy of chocolate boa BCH1. (C) Agarose gel confirming DNA purification, where distinct and well-defined bands are observed in each lane. In the case of the anaconda A sample, both the 250 bp and 300 bp bands are indicated.

Source: own elaboration.

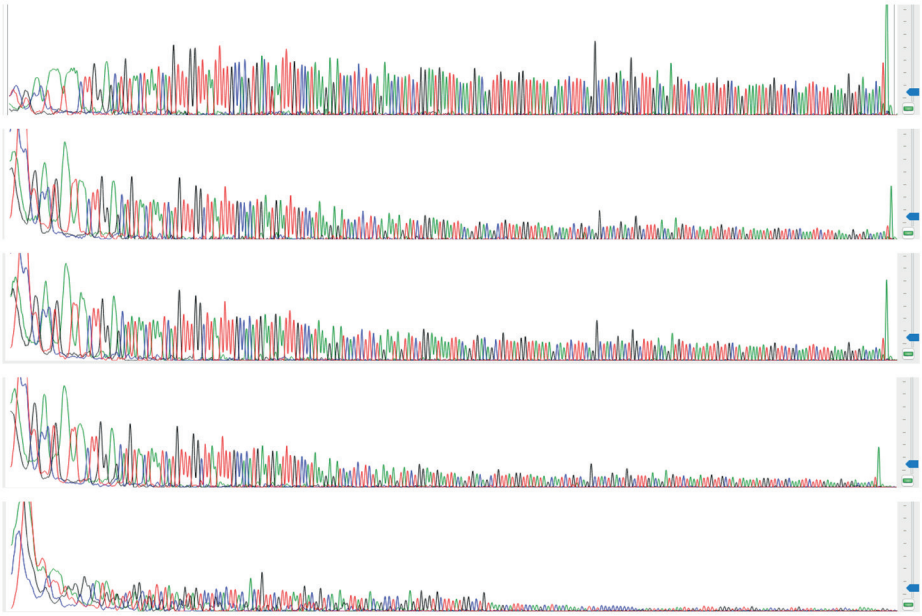

FIGURE 4. Chromatograms from Sanger sequencing reactions. From top to bottom: 220–250 bp band from the Boa constrictor BC2 sample, 220–250 bp band from the anaconda sample, 220–250 bp band from the chocolate boa BCH2 sample, 220–250 bp band from the liver sample obtained at necropsy of the chocolate boa BCH1, and 300 bp band from the anaconda sample.

Source: own elaboration.

bp bands may correspond to amplified mRNA sequences of snake origin that are not yet reported in current databases. Supporting this hypothesis, all individuals from the Boinae subfamily exhibited the same two bands (~220–250 bp and ~500 bp), which were absent in snakes outside this group. Conversely, all Pythonidae individuals consistently displayed highly similar bands—most prominently at 300, 700, and 1000 bp—that were not observed in Boinae snakes. In the false coral snake, the detected bands did not show similarities to those observed in either the Boinae or Pythonidae groups.

The specificity of the primers used in this study can therefore be questioned, as the preliminary results obtained were inconclusive. With regard to their design, the primers were deemed appropriate because they were developed from sequences within the S segment, which encodes the GP and NP genes and represents the most suitable target for Reptarenavirus detection (Argenta *et al.*, 2020; Hetzel *et al.*, 2021). However, although primers NP1 and NP2 showed alignment in PRIMER-BLAST primarily with the NP gene of species such as *R. giessenae* (University of Giessen Virus: UGV 1, 2, and 3) and *R. rotterdamense* (ROUT Virus: Boa Arenavirus NL), they did not align across the full diversity of *Reptarenaviruses* reported in the literature and databases, which is considerable (Hetzel *et al.*, 2021; Stenglein *et al.*, 2015). Given that the

assay did not yield conclusive evidence of *Reptarenavirus* detection, we conclude that the RT-PCR methodology employed here is not currently reliable, particularly in the absence of prior knowledge of the specific viral type or species circulating in snakes from the wildlife park. Although the observed bands corresponded to the expected amplicon size, these results should be regarded as false positives. For this reason, in the absence of a positive control, we recommend verifying band identity using an alternative method such as Sanger sequencing, as applied in this study. Even when positive controls are available, sequencing remains advisable due to the extensive genetic diversity of *Reptarenaviruses* and the experimental (rather than diagnostic) status of current PCR assays, with several "positive" results later disproven by sequencing (Hyndman *et al.*, 2019). To address the challenge posed by this high diversity, some studies have first conducted *de novo* sequencing of random samples from a population, followed by viral isolation and the development of RT-PCR assays specifically tailored to the *Reptarenavirus* types and species circulating in that population. These targeted assays can then be applied more reliably for large-scale screening (Argenta *et al.*, 2020; Baggio *et al.*, 2023; Thiele *et al.*, 2023).

Considering the above, several hypotheses can be proposed regarding the problem observed in the snakes from the RNBW. One possibility is that the snakes no longer harbor the virus but may have carried it in the past, as recent reports suggest that some *Boa constrictor* individuals can experience IBD subclinically and subsequently clear the infection (Dervas, 2024; Hetzel *et al.*, 2021; Thiele *et al.*, 2023). Another possibility is that the snakes are infected with a *Reptarenavirus* variant not detected by the RT-PCR assay used, given the extensive genetic variability of these viruses (Stenglein *et al.*, 2015) and the limited alignment of the primers with the full viral diversity currently available in databases. A further explanation is that blood samples may not represent the most suitable material for viral detection. In agreement with previous studies, higher viral loads are typically found in organs such as the liver, pancreas, and nervous tissue, while viremia is not consistently detectable in infected individuals (Baggio *et al.*, 2023; Moreira, 2010). This suggests that molecular testing should ideally be performed during peaks of clinical symptomatology. Finally, it is also possible that the snakes are affected by another virus, such as a member of the *Paramyxoviridae* family—particularly Ferlaviruses—which can produce clinical signs similar to IBD and are also associated with cytoplasmic inclusion bodies (Divers & Stahl, 2019; O'Rourke & Lertpiriyapong, 2015).

These results highlight the need for more precise and diversified methodologies for the detection of different *Reptarenaviruses*. This includes the use of a greater number of primers or assays such as multiplex PCR to simultaneously detect multiple types and species of *Reptarenaviruses* (Baggio *et al.*, 2023). Furthermore, the incorporation of positive controls is crucial, as they allow faster and simpler evaluation and standardization of newly designed PCR assays, although such controls were not available at the time of this study. Additional tools, such as real-time PCR, would enable in vivo quantification of viral genetic material in each sample. Finally, de novo sequencing and metatranscriptomic approaches could prove more effective in addressing and detecting the extensive viral diversity found in reptiles such as snakes.

As a final recommendation, whenever individuals in a snake population present neurological signs and evidence of cytoplasmic inclusion bodies in blood samples or biopsies, they should be maintained under indefinite quarantine or considered for euthanasia to protect the rest of the population. Both *Reptarenavirus* and Paramyxovirus (Ferlavirus) infections should be considered potential etiological agents, and efforts should be made to confirm the diagnosis using a robust molecular technique such as sequencing. Moreover, since the reptile mite (*Ophionyssus natricis*) has been proposed as a potential vector of the disease, strict hygiene and deparasitization protocols in snake enclosures are strongly recommended. In cases of mite infestation, a single oral administration of afoxolaner at a dose of 2.5 mg/kg has been demonstrated to be effective for mite elimination while remaining safe for snakes (Mendoza-Roldan *et al.*, 2023).

Conclusions

The RT-PCR assay designed in this study did not detect Reptarenavirus in the analyzed samples, and uncertainties remain regarding the identity of the bands observed. Therefore, this assay is not suitable for routine use in detecting Reptarenaviruses. For future studies, the inclusion of a positive control is recommended whenever available, as it facilitates the evaluation of the assay and its subsequent application. Nonetheless, band identity should always be confirmed by sequencing to avoid false positives.

With respect to primer design, it is concluded that the BLAST tool should be used as a guide to evaluate primer specificity; however, it must be considered that BLAST comparisons are limited to sequences reported in databases, which may not represent the full range of sequences present in a sample (e.g., host messenger RNA or DNA sequences not yet reported).

Finally, the health issues affecting snakes at the RNBW could be explained by several possible scenarios; therefore, further studies are required. These should incorporate a broader panel of primers and positive controls, or, when available, more robust diagnostic methods (e.g., de novo sequencing, multiplex PCR, qPCR, viral isolation, and direct observation). In addition, a greater diversity of sample types (biopsies, hemoglobin-free serum, urates, feces) should be included, while also considering alternative etiological agents such as Paramyxoviruses (Ferlaviruses).

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Conflict of interest

The authors declare no conflicts of interest.

Use of artificial intelligence

Artificial Intelligence (ChatGPT®) was used exclusively to adjust word count limits in the discussion and to verify compliance with bibliographic citation standards.

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