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Abstract: Boid inclusion body disease (BIBD) is an often fatal disease affecting mainly constrictor snakes. BIBD has been associated with infection, and more recently with coinfection, by various reptarenavirus species (family Arenaviridae). Thus far BIBD has only been reported in captive snakes, and neither the incubation period nor the route of transmission are known. Herein we provide strong evidence that co-infecting reptarenavirus species can be vertically transmitted in Boa constrictor. In total we examined five B. constrictor clutches with offspring ranging in age from embryos over perinatal abortions to juveniles. The mother and/or father of each clutch were initially diagnosed with BIBD and/or reptarenavirus infection by detection of the pathognomonic inclusion bodies (IB) and/or reptarenaviral RNA. By applying next-generation sequencing and de novo sequence assembly we determined the “reptarenavirome” of each clutch, yielding several nearly complete L and S segments of multiple reptarenaviruses. We further confirmed vertical transmission of the co-infecting reptarenaviruses by species-specific RT-PCR from samples of parental animals and offspring. Curiously, not all offspring obtained the full parental “reptarenavirome”. We extended our findings by an in vitro approach; cell cultures derived from embryonal samples rapidly developed IB and promoted replication of some or all parental viruses. In the tissues of embryos and perinatal abortions, viral antigen was sometimes detected, but IB were consistently seen only in the juvenile snakes from the age of 2 mo onwards. In addition to demonstrating vertical transmission of multiple species, our results also indicate that reptarenavirus infection induces BIBD over time in the offspring.

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Co-Infecting Reptarenaviruses Can Be Vertically Transmitted in Boa Constrictor

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ABSTRACT

Boid inclusion body disease (BIBD) is an often fatal disease affecting mainly constrictor snakes. BIBD has been associated with infection, and more recently with coinfection, by various reptarendnavirus species (family Arenaviridae). Thus far BIBD has only been reported in captive snakes, and neither the incubation period nor the route of transmission are known. Herein we provide strong evidence that co-infecting reptarendnavirus species can be vertically transmitted in boa constrictor. In total we examined five boa constrictor clutches with offspring ranging in age from embryos over perinatal abortions to juveniles. The mother and/or father of each clutch were initially diagnosed with BIBD and/or reptarendnavirus infection by detection of the pathognomonic inclusion bodies (IB) and/or reptarendnaviral RNA. By applying next-generation sequencing and de novo sequence assembly we determined the “reptarenvirome” of each clutch, yielding several nearly complete L and S segments of multiple reptarendnaviruses. We further confirmed vertical transmission of the co-infecting reptarendnaviruses by species-specific RT-PCR from samples of parental animals and offspring. Curiously, not all offspring obtained the full parental “reptarenvirome”. We extended our findings by an in vitro approach; cell cultures derived from embryonal samples rapidly developed IB and promoted replication of some or all parental viruses. In the tissues of embryos and perinatal abortions, viral antigen was sometimes detected, but IB were consistently seen only in the juvenile snakes from the age of 2 mo onwards. In addition to demonstrating vertical transmission of multiple species, our results also indicate that reptarendnavirus infection induces BIBD over time in the offspring.
Members of the genus *Reptarenavirus* are “newcomers” of the family *Arenaviridae* and have been associated with boid inclusion body disease (BIBD), an economically important, fatal disease of captive boid snakes. Recently, we and others observed that snakes with BIBD commonly harbour several S and L segments (arenaviruses have a bisegmented genome), which we refer to as co-infection. The above renders reptarenaviruses rather unique and a model for studying viral co-infection. We herein report that reptarenaviruses, and remarkably a whole set of co-infecting reptarenavirus species (based on the nucleotide difference in the L segment), can be transmitted vertically i.e. from parents to offspring. While the parental animals had BIBD, we did not find evidence of the intracytoplasmic inclusions characteristic to BIBD in the infected embryos and perinatal abortions. However, we could confirm the development of BIBD in offspring from an age of 2 months. Our findings further suggest that vertical transmission can, and likely has, significantly influence(d) the evolution of reptarenaviruses, since co-infection will allow reassortment of the viral genomes.
INTRODUCTION

Boid inclusion body disease (BIBD) is a transmissible, progressive and generally fatal disease of boid snakes. First described in the 1970s, BIBD subsequently emerged as a major problem in boid snake collections worldwide. Several genera of boid species have been reported as susceptible to the disease, but its prevalence among snakes as well as its potential occurrence in wild populations is yet unknown. Clinically, BIBD is highly variable particularly in boas, where affected animals can be free of clinical signs, die from secondary infections, or develop neurological signs. The latter are generally more pronounced in pythons. The hallmark of BIBD are the characteristic intracytoplasmic electron dense inclusion bodies (IB) that are found in most cell types. The pathogenesis of BIBD is not yet characterized, and both subclinical as well as chronic disease has been described.

A few years ago a novel group of arenaviruses were identified in and isolated from snakes with BIBD. Arenaviruses are negative-sense RNA viruses with two genome segments, L and S, which encode Z protein and RNA-dependent RNA polymerase, and glycoprotein precursor and nucleoprotein (NP), respectively. Strong evidence of the causative relationship between reptarenavirus infection and BIBD is provided by the ability of reptarenavirus isolates to induce the pathognomonic IB in an in vitro model, and by the fact that the IB contain or mainly consist of reptarenavirus NP. The identification of BIBD-associated arenaviruses led to the formation of a new genus, *Reptarenavirus*, in the family *Arenaviridae*, placing the previously known arenaviruses to another new genus, *Mammarenavirus*. More recently, we and others observed that snakes with BIBD often carry numerous distinct L and S segments, up to four S and 11 L segments were found in a single snake. The taxonomic classification of reptarenaviruses is currently under debate, and in this report we refer to the different L segments as representatives of different
reptarenavirus species (species share <76% identity in the L segment [8]). The genomes of
treptarenaviruses are highly variable [4, 5, 7, 10-13], as a consequence, the diagnosis of BIBD
still relies mainly on the detection of IB in cells in tissues or in blood smears by light
microscopy. A recent study screened a large panel of blood samples from captive boid
snakes, and found 19% of the snakes to be infected with reptarenavirus [14]. Among boa
constrictors, 41.5% were infected, and 87% of the infected snakes were clinically healthy
[14]. The authors also compared various detection techniques and found

immunohistochemistry (IHC) on blood cells, using a monoclonal anti-reptarenavirus NP
antibody, to provide results comparable to hematoxylin-eosin (HE) stained peripheral white
blood cells as the standard of diagnosis [14]. However, a few BIBD positive (3/25) samples
were negative in IHC [14], which provides further evidence of the high variability among
reptarenaviruses.

So far, the route of transmission and the incubation period of reptarenaviruses are unknown,
and direct contact or vector mediated transmission by snake mites (Ophionyssus natricis)
have been proposed [1, 15]. In line with the “transmission through a vector” hypothesis, we
recently reported the growth of reptarenaviruses also in arthropod cell lines [15]. Vertical
transmission is defined as any transfer of an infectious agent from one generation to the
next, including transmission through gametes (i.e. oocyte or spermatocyte), transplacental
transmission or perinatal infections [16]. Mammarenaviruses can be vertically transmitted in
their reservoir rodent hosts [17-20]. Prenatal infection plays an important role in arenavirus
maintenance, and, at least in the case of Lymphocytic choriomeningitis virus (LCMV),
Machupo virus (MACV) and Lassa virus (LASV), leads to chronic infection [21]. The vertical
transmission of BIBD from dam to offspring in both egg-lying and live-bearing snakes has
been considered by Chang and Jacobson [1]. Reptiles are divided into oviparous (egg layer)
or viviparous (live bearers) species. They represent an important phylogenetic intermedium
between anamniotes and amniote vertebrates, displaying all three embryonic membranes: the chorion, allantois and amnion. Viviparous snakes, including *Boa constrictor*, have a simple placenta that is responsible for gas exchange, and water and nutrient supply. A thin eggshell exists between foetal and maternal placenta but it deteriorates in late gestation, allowing direct contact between the foetal (chorioallantois) and maternal (uterine epithelium) placenta. Normally foetal and maternal epithelia remain intact and the maternal and fetal blood does not mix. Studies on the vertical transmission in reptiles are scarce and include only few viruses, such as equine encephalitis virus, adenovirus; Herpesvirus M and, very recently, Sunshinevirus.

We set up this study to determine whether reptarenaviruses can be vertically transmitted. For this purpose, five *Boa constrictor* clutches, represented by parental animals diagnosed with BIBD by traditional methods, or RT-PCR positive for reptarenavirus, and their offspring, ranging from embryos in the first trimester to 20-month-old juveniles, were examined. We applied next-generation sequencing (NGS) to identify the reptarenaviruses of each clutch, which we will refer to as the “reptarenavirome” throughout the manuscript. We utilized virus-specific RT-PCRs to confirm the reptarenavirus L and S segments identified by NGS. Primary cell cultures originating from the embryos served to evaluate the potential of the infecting viruses to induce IB formation and thereby also the disease.
RESULTS

*BIBD and reptarenavirus infection in the parents and offspring*

The diagnosis of BIBD is confirmed when the characteristic eosinophilic cytoplasmic IB are seen within cells. These IB contain abundant reptarenavirus NP which can be visualised by immunohistology (IH) \(^{[4,5,9]}\); RT-PCR can serve to confirm reptarenavirus infection. We verified the parental animals as BIBD positive and/or positive for reptarenavirus infection using histology and IH. The detection of the IB in cells in cytological and/or histological specimens is currently the widely accepted gold standard for the diagnosis of BIBD, since the IB are pathognomonic; IH confirms the presence of reptarenavirus NP in the cells \(^{[4]}\). For clutches 1 and 3-5 histology and IH were complemented by RT-PCR, which was set up at the time when only four reptarenaviruses were fully sequenced (referred to as “initial RT-PCR”); it targets the L segment of GGV, UHV, and Boa AV NL B3 (Table 1A and Fig. 1A, B). Interestingly, the blood of both parental animals in clutch 4 was RT-PCR-positive, but no IB were detected in blood cells. However, the subsequent post mortem analysis of the father revealed IB formation and expression of viral antigen in tissues, confirming BIBD (Table 1).

For clutch 1, comprised of seven embryos in late first trimester (age determined based on the body length of 15 to 17 cm), five embryos were processed for (immuno)histological examination. These did not exhibit IB formation, but exhibited weak reptarenavirus antigen expression in occasional cells in brain, liver and kidneys (Fig. 1C). The remaining two embryos (E1.6 and E1.7, Table 1) were used to establish primary cell cultures. These showed viral antigen expression, but no distinct IB formation (Fig. 1D; Table 1). The initial RT-PCR showed the presence of reptarenavirus RNA in the mother and embryos E1.1, E1.6, and E1.7. (Table 1A).
For clutch 2 (early first trimester embryos with a body length of 5-6 cm), similar results were obtained. Two of the three embryos (E2.1 and E2.2) were used to establish primary cell cultures, which also showed viral antigen expression but no IB. The cell cultures remained persistently infected throughout the study period as confirmed by the expression of viral RNA and antigen. The third embryo (E2.3) was processed for histology and did not exhibit IB but showed occasional weak viral antigen expression in the brain (Table 1A).

Clutch 3 comprised five animals, three of which had been perinatally aborted (PNA3.1 to 3.3). Two of these (PNA3.1 and 3.2) were tested reptarenavirus RNA positive, using the initial RT-PCR on the brain, and one (PNA3.2) exhibited IB and reptarenavirus antigen in the tissues (Fig. 1E, F). The remaining two animals (J3.1 and 3.2) were euthanized as juveniles two months later. Both were tested positive by the initial RT-PCR on the brain and one also exhibited IB and reptarenavirus antigen in tissues (Table 1A).

The two perinatal abortions of clutch 4 were shown to be infected, using the initial RT-PCR, but did not exhibit IB formation or reptarenavirus antigen expression.

Clutch 5 comprised 21 animals. Of the seven juveniles euthanized at the age of eight months, six were diagnosed with BIBD, based on the detection of IB and viral antigen in all examined tissues (Fig. 1G, H), and three of these (3/6) were found positive in the blood by the initial RT-PCR. At the time of euthanasia the samples were collected purely for diagnostic purposes, and unfortunately no samples were stored for RNA isolation. The remaining (1/7) animal (J5.5) was negative in all these tests. Another 11 siblings were euthanized at the age of 12 months. In nine of these, BIBD was confirmed, with the presence of IB and reptarenavirus antigen in tissue and blood cells and a positive result in the initial RT-PCR. Two (2/11) (J5.8, J5.11) were BIBD-negative, but RT-PCR positive in the brain (Table 1A). The last four (4/21) animals were kept by the breeder until they were euthanized at the age of 18 mo (n=2) and 20 mo (n=2) due to the breeder’s concern that they
suffered from BIBD. These all tested positive for BIBD by histology, IH and initial RT-PCR (Table 1A).

Confirmation of the vertical transmission by next-generation sequencing (NGS) of total RNA isolated from the parents and/or offspring.

The primers used for RT-PCR in the preliminary screening were designed for the detection of a subset of reptarenaviruses (GGV, UHV, and Boa AV NL B3) at a time when only four reptarenaviruses were known. Subsequently, we and others\(^{10,11}\) observed that snakes with BIBD are often co-infected with multiple reptarenavirus species. Therefore, we decided to utilise NGS for further analyses. The NGS study included the first four clutches, but was limited to the animals of which frozen material was available (Table 1A). We removed the reads matching a known snake genome (Python bivitattus) from the NGS data and performed *de novo* genome assembly. The generated contigs were checked using BLAST (Basic Local Alignment Search Tool, https://blast.ncbi.nlm.nih.gov/Blast.cgi), and although occasional hits to bacterial sequences were identified in some samples, only reptarenavirus sequences were consistently recovered. Similarly to our earlier observation\(^{11}\), several full-length or almost full-length (at maximum some 200-300 nt missing) reptarenavirus L and S segments were recovered from the parental samples. The coverages (Bowtie 2,\(^{29}\) of the reptarenavirus L and S segments derived from the parental animals were >10 (lower coverage at the very last ~50 nts) (supplementary Table 2). In parental animals from breeder 1 (clutches 1 and 3), the following results were obtained: The mother of clutch 1 was positive for six L (Aurora borealis virus-4, ABV-4, GenBank accession KX527594; Tavallinen suomalainen mies virus-1, TSMV-1, KX527595; Hans Kompis virus-1, HKV-1, KX527596; Keijut pohjoismaissa virus-1, KePV-1, KX527597; Bis spöter virus-1, BSV-1, KX527598; Suri Vanera virus, SVaV-2, KX527599) and two S (S6-like, KX527580; S5-like, KX527581) segments, and the mother of clutch 3 was positive
KX527587; Kuka mitä häh virus-1, KMHV-1, KX527588; KePV-1, KX527589; University of Helsinki virus-4, UHV-4, KX527590; TSMV-2, KX527591; ABV-4, KX527592; Grüetzi mitenand virus-1, GMV-1, KX527593) and two S (S6-like, KX527578; S5-like, KX527579) segments. Curiously, the brain of the father of clutch 4 was positive for only one pair of L (TSMV-2, KX527582) and S (TSMV-2, KX527575) segments, whereas no reptarenavirus genomes were recovered by NGS from the mother despite clear evidence of BIBD (Table 1A). The mother of clutch 2 owned by breeder 2 was positive for four L (ABV-3, KX527583; Kaltenbach virus-1, KaBV-1, KX527584; SVaV-1, KX527585; UHV-3, KX527586) and two S (ABV-2, KX527576; University of Giessen virus-1-like, UGV-1-like, KX527577) segments, whereas no reptarenavirus genomes were recovered from the serum of the father, whose blood cells were also found negative for IB in the cytological examination, providing further evidence that he was indeed not infected at all. The NGS results for the different clutches are summarized in Table 1B and a phylogenetic tree of the de novo assembled L and S segments with database sequences is shown in Fig. 2A and B. The phylogeny indicates that the reptarenaviromes of the two snake collections (which never exchanged animals; personal communication) share some common species but also comprise unique viruses. Initially de novo assembly was attempted for several embryos (E1.1, E1.2, E1.7, E2.1 - E2.3), however, this approach was not successful, likely due to inefficient removal of the genomic background during NGS library preparation and low amounts of viral RNA. Instead, we used the reptarenavirus genomes obtained from the parental animal to “fish out” i.e. to map the matching reads from the embryos, an approach we then also took for clutches 3 and 4. However, only scattered reads matching the parental viruses could be recovered from the NGS data for most embryos (supplementary Table 2). Thus we decided to confirm the NGS findings by conventional RT-PCR using virus species-specific (VSS) primers,
primers of our previous study [11] and primers designed based on the de novo assembled arenavirus genomes (primer sequences in supplementary Table 1). For most clutches we also included additional samples, from tissues or cell cultures generated from the embryos, into the RT-PCR analysis (Table 1B and C).

For the three embryos of clutch 1, the mapping yielded reads matching five (E1.1), two (E1.2) and three (E1.7) of the six L segments and both S segments (all embryos) identified in the mother. For the primary cell culture of E1.7, the reads each covered the entire segments, which might be a consequence of the higher virus content in the supernatant compared to the tissues which were examined for E1.1 and E1.2. The VSS RT-PCRs confirmed the presence of several to all parental L and S segments in the embryonal tissues (E1.1 and E1.2) and cultured brain cells (E1.6 and E1.7) (Table 1B and 1C).

For clutch 2, reads matching two L and both S segments were identified by mapping the NGS data of E2.1 (kidney cell culture), E2.2 and E2.3 (both tissue homogenates) against parental viruses (supplementary Table 1). The VSS RT-PCRs confirmed the NGS findings and identified the parental L and S segments also in homogenates of salpinx and placenta and in cultured cells from umbilicus, placenta and organs (Table 1B and 1C).

For clutch 3, we identified reads matching three L and two S segments of the maternal viruses for two perinatal abortions (PNA3.1 and 3.3) and reads matching each two L and S segments for the third (PNA3.2) by the mapping approach. VSS RT-PCRs on samples from several organs (brain, kidney, liver) confirmed the NGS findings. They also identified maternal L and S segments in the liver and kidney of the juvenile snakes euthanized at the age of 2 months (Table 1B and 1C).

For clutch 4, the mapping approach yielded a few reads matching both the L and S segment of the virus identified in the father in one perinatal abortion (PNA4.1), and for the second (PNA4.2), only a single read matching the L segment. Since the subsequent VSS RT-PCR
of the PNA samples yielded only a weak reaction for the TSMV-2 L segment, we then
applied all L segment primers available from the different viruses to RNA extracted from
paternal blood and lung, and from the maternal blood sample. Curiously, while the brain of
the father remained positive for only a single virus, the blood contained a further 7
reptarenavirus L segments, three of which were also found in the maternal blood. VSS RT-
PCRs then identified several paternal L and S segments in the tissues of both perinatal
abortions (Table 1B and 1C).
Since the results obtained from clutches 1, 3 and 4 suggested that we had characterized the
“reptarenavirome” of breeder 1’s collection, we did not perform NGS for clutch 5, but tested
the father and several of his 12-month-old juvenile offspring, which were in the majority
confirmed to suffer from BIBD based on the presence of viral IB and viral antigen in
tissues, with all L and S segment VSS RT-PCRs of the present and an earlier study [11]. The father was positive for four of these viruses, and the juveniles were all found to carry
at least two of their father’s L segments (Table 1B and 1C). The results for the L segment
VSS RT-PCRs for each clutch are summarized in Figure 3. The raw data for VSS RT-PCRs
are shown in Supplementary Figures 1-5 (S1 Figure, S2 Figure, S3 Figure, S4 Figure and S5
Figure).
DISCUSSION

So far, studies on the transmission of reptarenaviruses are scarce, and transmission via direct contact, through droplets or aerosols, or via vectors has been discussed\(^1,2\). In this study on naturally infected captive animals we combined classical and more modern techniques and could demonstrate that reptarenaviruses and BIBD can be vertically transmitted. The study included five *Boa constrictor* clutches with BIBD-positive parental animals, and by NGS combined with *de novo* genome assembly we could retrieve nearly complete reptarenavirus L and S segments in three of the four studied parental snakes. Because the different L segments identified in the parental animals were <76% identical to each other, we interpreted their identification as evidence of reptarenavirus co-infection. We could further show that co-infecting reptarenaviruses are often co-transmitted vertically from parents to offspring. By combining NGS and virus species-specific (VSS) RT-PCRs we could confirm the vertical transmission(s) and show that the offspring retains co-infecting viruses over a long period of time, i.e. for at least 12 months after birth.

Currently the strongest evidence of reptarenaviruses being the causative agents of BIBD is the fact that the IBs pathognomonic to BIBD\(^2\) consist mainly, if not solely, of reptarenavirus NP\(^4,5,9,14\). Although this does not rule out the possibility of another, yet unidentified, microbe [for example an (endogenous) retrovirus] contributing to the development of the disease, it clearly demonstrates that reptarenavirus infection is a prerequisite for BIBD. In the embryos, reptarenavirus infection was not associated with IB formation; however, viral antigen was found in occasional cells in brain, liver and kidneys. Furthermore, primary cell cultures derived from embryos of BIBD positive mothers promoted (part of) the maternal reptarenaviriome and also showed viral antigen expression. IB formation was seen in older offspring, first in one of the PNA, consistently in all virus
genome-positive juveniles from 2 months of age, confirming that reptarenavirus infection in vivo does indeed provoke all the characteristics of BIBD.

Vertical transmission occurs in the reservoir hosts of many arenaviruses. For example, LCMV and MACV can be transmitted transovarially \[17, 18\] and/or transplacentally \[19\]. Additionally, infection through semen or maternal blood has been suggested for MACV and Latino virus \[20\]. Prenatal infection plays an important role in virus maintenance, since for some arenaviruses (LCMV, MACV, and LASV) it may lead to chronic infections \[21\].

For reptarenaviruses, the precise mode of vertical transmission is not yet known, but our study provides evidence that the viruses of both mother and father can be passed to the offspring, and that the transmission can occur already early in gestation. We were able to isolate viruses also from cell cultures originating from placenta, salpinx, and umbilicus. Since the Boa constrictor embryo does not get into contact with the maternal blood, this indicates that transmission from the mother could also result from contact between maternal tissues and the chorioallantois. However, more detailed studies on the reproductive tract of snakes with BIBD are needed to elucidate the exact mechanisms of transmission from both the maternal and paternal animal.

The convention among snake breeders that also both breeders in our study followed is that the neonates are removed from the mother’s cage within a few hours. The clutch is then housed separately until the first shedding at 6-12 days of age, after which the animals are separated and housed in individual cages \[30\]. This, together with the strict hygiene rules that are applied, does not exclude transmission of viruses between siblings during their first days of life, but renders horizontal infection unlikely thereafter.

It was overall surprising to see how many offspring exhibited reptarenavirus infection without evidence of IB formation or viral antigen expression (4/5 perinatal abortions, one 2-month-old juvenile) or without IB formation and only occasional cells expressing viral
antigen (all tested embryos, two 12-month-old juveniles), i.e. BIBD. Also, the fact that we found BIBD-negative animals to carry reptarenaviral RNA in the blood suggests that viremia may occur frequently, not only in association with the disease, but also in seemingly healthy animals. However, light microscopy and IH are comparatively insensitive methods, and thus the above findings could also be due to low level viral replication. Alternatively, our findings could indicate that reptarenavirus infection has a long incubation period, and that both endogenous and exogenous factors can influence the development of BIBD. It has recently been suggested that transient reptarenavirus infections can occur [31]. Although we cannot disprove this assumption, the fact that the vast majority of juvenile offspring from snakes with BIBD in our study eventually developed BIBD suggests that at least prenatal reptarenavirus infections generally persist. We recently observed that snakes with BIBD rarely exhibit anti-reptarenavirus antibodies [32]. This could indicate that prenatal infection results in tolerance to reptarenaviruses, allowing persistent infection. Chang and co-workers recently reported that the vast majority of reptarenavirus infected and BIBD positive boa constrictors are clinically healthy [14], which would be in line with the above hypothesis. Further studies are required to show if the hypothesis is correct and what determines the subsequent IB formation.

Our observation on the vertical transmission of co-infecting viruses sheds light on the potential evolution of reptarenaviruses. The research field of “reptarenavirology” is fairly young, and the taxonomical classification scheme of these viruses is yet to be determined. After the most recent report from the arenavirus study group of the International Committee on Taxonomy of Viruses (ICTV) [8] our group and the group of Stenglein and co-workers reported a multitude of complete L and S segments identified by NGS in tissues of snakes with BIBD [10, 11]. Both groups also observed a seemingly unbalanced ratio of L and S segments in a single individual, similarly to what we report herein. These findings are the
challenge for the classification of reptarenaviruses. For the present report we followed the ICTV and considered the different reptarenavirus L segments to derive from different reptarenavirus species when their nucleotide sequence identity was below 76%. We took this approach, because currently not all of the information required to fulfil all criteria of a virus species are available. We also hypothesize that in the past, i.e. before multiple cross-species transfers (between and from the unknown reservoir hosts), each L and S segment pair formed a definite, classifiable species. For mammarenaviruses it has been reported that the persistent infection of cell cultures with one mammarenavirus excludes the replication of homologous and antigenetically related viruses, but enables the growth of non-related mammarenaviruses. Similarly, we have only identified L segments of different reptarenavirus species (based on the criteria above) in snakes with BIBD. Assuming that there is (or was) a reservoir host for each reptarenavirus species, it can be hypothesised that, with more relaxed hygiene regimens, housing different snake species in the same facilities has enabled cross-species mixing of the viruses. Co-infection might then have enabled the mixing of L and S segments, and reassortment, and vertical transmission of these persistently infecting viruses may have contributed to the plethora of reptarenaviruses that we now detect in captive boid snakes. The apparent existence of more viral L than S segments might be related to the fact that the S segment harbours the viral glycoproteins. As these are essential for host cell entry, the S segment that guarantees the most efficient gene transfer might be enriched during co-infections. The selection pressure on the S segment may further be enforced by the functions of the NP in viral replication. If the L and S segments could pair more or less freely with each other, the selection pressure on the L segment (harbouring the RNA dependent RNA polymerase and the viral matrix protein) would be less strong. It is also possible that more than a single pair of L and S segments are packed inside the virion, which would render the taxonomical classification of
reptarenaviruses even more complex. Currently there is no data on the factors enabling or
disabling the pairing of different L and S segments. Since protein-protein interactions,
among other factors, contribute to the formation of progeny viruses and cell-cell
transmission, we speculate that L and S segment pairing would not occur in a completely
random fashion (otherwise one would expect to have roughly equal numbers of known L
and S segments). Further studies are needed to tackle these questions and to prove or
disprove the above hypothesis, which only represents a simplified version of reality.

In order to avoid infection and/or spreading of the disease within a collection, it would be
essential to know all the factors behind reptarenavirus transmission. A six-month quarantine
is generally recommended before a new animal is released into a collection, but whether this
is sufficient to avoid reptarenavirus transmission is so far unknown [35]. The results that we
obtained from clutch 5 indicate that it can take several months before a prenatally infected
snake exhibits definite signs of BIBD. In any case, our results demonstrate that animals with
BIBD/reptarenavirus infection should not breed, since the likelihood of offspring to become
infected is high.
MATERIALS AND METHODS

Ethics statement

All animals included into the study were snakes that were submitted by their owners to the Department of Veterinary Pathology, Vetsuisse Faculty, University of Zurich, Switzerland. They were euthanized according to ASPA, Animals (Scientific Procedures) Act 1986, schedule 1 (appropriate methods of humane killing, http://www.legislation.gov.uk/ukpga/1986/14/schedule/1) procedure and a full diagnostic post mortem examination was performed. Tissue samples from the dead animals were subjected to the different tests with owners' consent. The owners consented both to the euthanasia and the use of collected samples in this study. Because of suspected BIBD no ethical permissions were required for euthanasia nor the diagnostic-motivated necropsies (both routine veterinary purposes).

Animals and sampling

The study was performed on five B. constrictor clutches from two breeders residing in Switzerland. The two breeders confirm that they have never exchanged animals with each other. All animals were examined for diagnostic purposes, i.e. BIBD diagnosis, upon the owners’ request, which was undertaken on a blood smear and/or by a full post mortem examination. Parental animals that were not euthanized were bled from the tail vein or by cardiac puncture to prepare a blood smear. For necropsy, animals were narcotized with CO$_2$ followed by decapitation and immediate destruction of the brain by longitudinal sectioning. Immediately after euthanasia, a full post mortem examination was performed.

The following B. constrictor snakes were examined (Table 1); clutch 1: a BIBD-positive (blood smear) pregnant female (euthanized due to emaciation and poor general health) with seven embryos in the first third of gestation; clutch 2: a BIBD-positive pregnant female (euthanized due to the owner's suspicion of illness and BIBD) with three embryos in the
first third of gestation, the father was subsequently tested on blood smears; clutch 3: three
perinatal abortions and two siblings euthanized at the age of two months for diagnostic
purposes, blood tested from the mother for BIBD diagnosis; clutch 4: two perinatal
abortions, blood tested from mother and father for BIBD diagnosis; clutch 5: 22 juveniles
(seven euthanized at the age of eight months, eleven at 12 months, two at 18 months, two at
20 months) for BIBD diagnosis due to positivity of the father, euthanasia and post mortem
evacuation of the father due to emaciation and chronic pyogranulomatous bacterial rhinitis.
The clutch had been separated from the mother within 8 h after birth and individual animals
housed separately since after the first shedding at 6-12 days of age.
From all necropsied animals, tissue samples were collected from a range of organs (brain,
heart, lung, liver, pancreas, kidney, spleen and – in selected cases – spinal cord), fixed in
10% buffered formalin, and routinely paraffin wax embedded for histological and
immunohistological examinations. Selected embryos were fixed and paraffin wax embedded
in toto, others were subjected to RNA extraction and/or establishment of cell cultures (Table
1). For adult and juvenile snakes blood smears were prepared and air-dried for cytological
examination, and the remaining blood was centrifuged at 1,000 x g for 5 min to separate
serum and blood cells. The samples for RNA extraction and/or virus isolation were collected
and frozen freshly at -80 °C without fixative or processed immediately.

Cytological, histological, and immunohistological examination
Blood smears were stained with May-Grünwald-Giemsa and a cytological examination was
performed to determine the presence of the pathognomonic cytoplasmic IB within blood
cells, as previously described. From paraffin blocks, consecutive sections (3-5 μm) were
prepared, stained with hematoxylin-eosin (HE) for the identification of the cytoplasmic IB,
and subjected to immunohistological staining, using a rabbit anti-UHV NP antibody for
the demonstration of reptarenavirus antigen, as described earlier [4]. Consecutive sections incubated with a non-reactive rabbit polyclonal antiserum served as negative controls.

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Cell cultures

From selected embryos (Table 1), samples of brain, heart, liver, kidney, umbilical cord and/or placenta were aseptically collected and subjected to tissue culture (30 °C, 5% CO₂), as described [4]. After passaging of the established cell cultures, aliquots of the cultures (cell-rich supernatants) were frozen at -80 °C (Table 1). The established cell cultures were analysed at each passage and remained persistently infected throughout the study. The cell culture supernatants from the established cell cultures were used to inoculate permanent boid kidney cell cultures (I/1Ki [4]), for virus identification by NGS, and to prepare cell pellets for formalin fixation and paraffin wax embedding, followed by immunohistology for the detection of reptarenavirus antigen, as previously described [4]. Cell pellets prepared from uninfected control cells served as the negative, and cells infected with the UHV isolate [4] (containing both ABV-1 and UHV-1 [11]) as the positive controls.

Sample preparation and reverse transcription-polymerase chain reaction (RT-PCR)

RNA was extracted from tissue samples with TRIzol or Trizol LS reagent (Life Technologies) utilizing mechanical homogenization with MagNA Lyser (Roche) following the manufacturer’s protocol. From cell culture supernatants (Table 1), RNAs were isolated with the QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer’s instructions.

RNA isolation from blood samples (Table 1) was performed according to a modified protocol for avian blood [36]. Briefly, 100 µl of centrifuged, cell-enriched blood was mixed with 900 µl of TRIzol® (or 250 µl of blood and 750 µl of Trizol LS) and homogenized through pipetting. After addition of chloroform and separation of the RNA containing phase
by centrifugation (15 min, 12,000 x g, 4 °C) the RNA was purified with the QiaGEN
RNeasy Mini Kit (Qiagen) following the manufacturer’s protocol for RNA clean up.
The cDNAs were transcribed with random primers (1/6-1/10 of the isolated RNA was used
as template) using either RevertAid Transcriptase or RevertAid Premium Transcriptase
(both from Thermo Fisher Scientific), following the manufacturer’s recommendations for
RNA transcription with random hexamer primers.
Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific) with 2 μl of
cDNA as the template was utilized in the “initial” PCR and 1 μl in the VSS PCR reactions.
The “initial” PCR, applied prior to obtaining the NGS data, was run using primers (Fwd 5’-
GAGCACGTCCTGTGTGTGT-GTGGTTGTGTATGGGAGAGG -3’ and Rev 5’-GTGGTTGTGTATGGGAGAGG -3’)
targeting an approximately 170 bp long fragment of the L segment of GGV (1199-1367),
UHV (1201-1369), and Boa AV NL3 (1191-1359) in PCR amplifications with Phusion
Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific) using the following cycling
conditions: 1. initial denaturation 15 s at 98 °C, 2. denaturation 1 s at 98 °C, 3. annealing 5 s
at 60 °C, 4. extension 5 s at 72 °C, steps 2 to 4 were repeated 38 times until final extension
of 1 min at 72 °C. For Sanger sequencing (performed by the DNA sequencing core facility
of Medicum, University of Helsinki, Finland, or by Microsynth, Switzerland), the products
of the “initial” RT-PCR were purified using either QIAquick PCR purification Kit (Qiagen)
or QIAquick Gel Extraction Kit (Qiagen), both according to the manufacturer’s instructions
After NGS and de novo assembly (see below), virus species-specific (VSS) primers were
designed and used to confirm the presence of viruses identified by NGS in parents and
offspring. The VSS RT-PCRs (both the S and L segment primers are listed in
supplementary Table 1) with Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher
Scientific) and 1 μl of cDNA were run using the following cycling conditions: 1. initial
denaturation 15 s at 98 °C, 2. denaturation 1 s at 98 °C, 3. annealing 5 s at 60 °C, 4.
extension 7 s at 72 °C, steps 2 to 4 were repeated 38 times until final extension of 1 min at
72 °C. The products were analyzed by agarose gel electrophoresis (gel strength 1.5-1.75%) with GelRed nucleic acid stain (Biotium) pre-cast to gels, the visualization of bands was
done under UV-light. Samples from various tissues of different animals served as the
negative controls for each primer pair. The raw data for VSS RT-PCRs are shown in
Supplementary Figures 1-5 (S1 Figure, S2 Figure, S3 Figure, S4 Figure and S5 Figure).

Next-generation sequencing (NGS), de novo assembly, and phylogenetics

The purified RNAs were treated with DNAse I (Fermentas), and re-purified using the
GeneJET RNA purification kit (Thermo Fisher Scientific). The RNA was further cleaned
using the Rib-Zero Gold rRNA Removal Kit for Epidemiology (Illumina) according to the
manufacturer’s protocol. The indexed (New England Biolabs) NGS libraries were prepared
using the NEBNext Ultra RNA Library Preparation Kit (New England Biolabs) following
the manufacturer’s instructions. The library quantification was done using the NEBNext
Library Quant Kit for Illumina (New England Biolabs), and 291-bp paired-end reads of the
pooled libraries were sequenced on an Illumina MiSeq (Illumina) using the MiSeq Reagent
Kit v3 (Illumina). Removal of reads matching the host genome and de novo sequence
assembly were performed using MIRA version 4.0.2. (http://mira-assembler.sourceforge.net/) on CSC (IT Center for Science Ltd., Finland) Taito
supercluster. Chipster v.3.1.0. was applied for the generation of subsets and any other
handling of the data [37]. The reptarenavirus genomes de novo assembled from the parents’
samples were used to map the reads matching reptarenaviruses from the offspring samples
in Unipro UGENE 1.14.2. [38] utilizing the Bowtie2 [29] tool. The raw NGS data is publicly
available through sequence read archive (SRA) under object IDs 5921116 to 5921133 with
respective URLs http://www.ncbi.nlm.nih.gov/sra/5921116 to http://www.ncbi.nlm.nih.gov/sra/5921133.

Phylogenetic analyses were performed with the newly recovered sequences combined with representative reptarenavirus sequences obtained from the NIAID Virus Pathogen Database and Analysis Resource (ViPR) \[39\] through the web site at http://www.viprbrc.org/.

Complete S segment nt sequences (S6-like clutch 1, KX527580; S5-like clutch 1, KX527581; ABV-2 clutch 2, KX527576; UGV-1 clutch 2, KX527577; S6-like clutch 3, KX527578; S5-like clutch 3, KX527579; TSMV-2 clutch 4, KX527575; ABV-1, KR870010; ABV-2, KR870018; Boa AV NL3, NC_023761; CASV, NC_018481; GGV, NC_018483; UHV-1, KR870011; UHV-1 (Hetzel et al.), NC_023766; UHV-2, KR870016; UHV-3, KR870019; UGV-1, KR870012; UGV-2, KR870015; UGV-3, KR870013; UGV-4, KR870014; S1, KP071530; S2, KP071541; S3, KP071630; S4, KP071474; S5, KP071599; S6, KP071673; S6A, KP071502; S6B, KP071501; S7, KP071578; S8, KP071509; S9, KP071671; S10, KP071558; S11, KP071559) were aligned with ClustalX \[40\]. The nt sequences (abbreviation, accession code: ABV-4 clutch 1, KX527594; TSMV-1 clutch 1, KX527595; HKV-1 clutch 1, KX527596; KePV-1 clutch 1, KX527597; BSV-1 clutch 1, KX527598; SVaV-2 clutch 1, KX527599; ABV-3 clutch 2, KX527583; KaBV-1 clutch 2, KX527584; SVaV-2 clutch 1, KX527585; UHV-3 clutch 2, KX527586; SVaV-2 clutch 3, KX527587; KMHV-1 clutch 3, KX527588; KePV-1 clutch 1, KX527589; UHV-4 clutch 3, KX527590; TSMV-2 clutch 3, KX527591; ABV-4-clutch 3, KX527592; GMV-1 clutch 3, KX527593; TSMV-2 clutch 4, KX527582; ABV-1, KR870021; ABV-2, KR870033; ABV-3, KR870025; Boa AV NL3, NC_023762; CAS virus, CASV, NC_018484; Golden Gate virus, GGV, KP071475; HKV-1, KR870028; SVaV-1, KR870024; TSMV-1, KR870026; UHV-1, KR870020; UHV-1 (Hetzel et al.), NC_023765; UHV-2, KR870030; UHV-3, KR870032; UHV-4, KR870027; UGV-1, KR870022; UGV-2, KR870029; UGV-3,
KR870023; L1, KP071529; L2, KP071475; L3, KP071523; L4, KP071488; L5, KP071489;
L6, KP071492; L7, KP071477; L8, KP071511; L9, KP071563; L10, KP071503; L11,
KP071512; L12, KP071550; L13, KP071574; L14, KP071562; L15, KP071551; L16,
KP071614; L17, KP071547; L18, KP071481; L19, KP071548; L20, KP071564; L21,
KP071478; L22, KP071476) coding for the RNA-dependent RNA polymerase were aligned
using amino acid translation guidance in Translator X \cite{41} with the MAFFT algorithm. The
GTR (general time reversible) model was used for nucleotide substitutions. Phylogenetic
trees were reconstructed by the maximum-likelihood method in MEGA 6.06 with 1,000
bootstrap replicates.
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REFERENCES

1. Chang L, Jacobson ER. (2010) Inclusion body disease, A worldwide infectious disease of boid snakes: A review. Journal of Exotic Pet Medicine 19(3): 216-225.

2. Schumacher J, Jacobson ER, Homer BL, Gaskin JM. (1994) Inclusion body disease in boid snakes. Journal of Zoo and Wildlife Medicine 25(4): pp. 511-524.

3. Vancraeynest D, Pasmans F, Martel A, Chiers K, Meulemans G, et al. (2006) Inclusion body disease in snakes: A review and description of three cases in boa constrictors in Belgium. Vet Rec 158(22): 757-760.

4. Hetzel U, Sironen T, Laurinmaki P, Liljeroos L, Patjas A, et al. (2013) Isolation, identification, and characterization of novel arenaviruses, the etiological agents of boid inclusion body disease. J Virol 87(20): 10918-10935.

5. Stenglein MD, Sanders C, Kistler AL, Ruby JG, Franco JY, et al. (2012) Identification, characterization, and in vitro culture of highly divergent arenaviruses from boa constrictors and annulated tree boas: Candidate etiological agents for snake inclusion body disease. MBio 3(4): e00180-12.

6. Carlisle-Nowak MS, Sullivan N, Carrigan M, Knight C, Ryan C, et al. (1998) Inclusion body disease in two captive australian pythons (morelia spilota variegata and morelia spilota spilota). Aust Vet J 76(2): 98-100.

7. Bodewes R, Kik MJ, Raj VS, Schapendonk CM, Haagmans BL, et al. (2013) Detection of novel divergent arenaviruses in boid snakes with inclusion body disease in the Netherlands. J Gen Virol 94(Pt 6): 1206-1210.
8. Radoshitzky SR, Bao Y, Buchmeier MJ, Charrel RN, Clawson AN, et al. (2015) Past, present, and future of arenavirus taxonomy Arch Virol 160(7): 1851-1874.

9. Chang LW, Fu A, Wozniak E, Chow M, Duke DG, et al. (2013) Immunohistochemical detection of a unique protein within cells of snakes having inclusion body disease, a world-wide disease seen in members of the families boidae and pythonidae PLoS One 8(12): e82916.

10. Stenglein MD, Jacobson ER, Chang LW, Sanders C, Hawkins MG, et al. (2015) Widespread recombination, reassortment, and transmission of unbalanced compound viral genotypes in natural arenavirus infections PLoS Pathog 11(5): e1004900.

11. Hepojoki J, Salmenpera P, Sironen T, Hetzel U, Korzyukov Y, et al. (2015) Arenavirus coinfections are common in snakes with boid inclusion body disease J Virol 89(16): 8657-8660.

12. Bodewes R, Raj VS, Kik MJ, Schapendonk CM, Haagmans BL, et al. (2014) Updated phylogenetic analysis of arenaviruses detected in boid snakes. J Virol 88(2): 1399-1400.

13. Hetzel U, Sironen T, Laurinmaki P, Liljeroos L, Patjas A, et al. (2014) Reply to "updated phylogenetic analysis of arenaviruses detected in boid snakes". J Virol 88(2): 1401-13.

14. Chang L, Fu D, Stenglein MD, Hernandez JA, DeRisi JL, et al. (2016) Detection and prevalence of boid inclusion body disease in collections of boas and pythons using immunological assays Vet J 218: 13-18.
600 15. Hepojoki J, Kipar A, Korzyukov Y, Bell-Sakyi L, Vapalahti O, et al. (2015) Replication of boid inclusion body disease-associated arenaviruses is temperature sensitive in both boid and mammalian cells J Virol 89(2): 1119-1128.

603 16. Studdert VP, Gay CC, Blood DC. (2011) Saunders comprehensive veterinary dictionary 4th edition direct textbook. : 1344.

605 17. Johnson K, Webb PA, Justines G. (1973) Biology of tacaribe-complex viruses. In: Anonymous Lymphocytic choriomeningitis virus and other arenaviruses. : Springer. pp. 241-258.

608 18. Mims CA. (1966) Immunofluorescence study of the carrier state and mechanism of vertical transmission in lymphocytic choriomeningitis virus infection in mice J Pathol Bacteriol 91(2): 395-402.

609 19. Mims CA. (1981) Vertical transmission of viruses Microbiol Rev 45(2): 267-286.

610 20. Webb PA, Justines G, Johnson KM. (1975) Infection of wild and laboratory animals with machupo and latino viruses Bull World Health Organ 52(4-6): 493-499.

614 21. Childs JE, Peters CJ. (1993) Ecology and epidemiology of arenaviruses and their hosts. In: Anonymous The Arenaviridae. : Springer. pp. 331-384.

616 22. Norris D, Lopez K. (2010) Hormones and reproduction of vertebrates, volume 3, 1st edition. : 432.

618 23. Blackburn DG, Stewart JR. (2011) Viviparity and placentation in snakes. In: Aldrich RD, Sever DM, editors. Reproductive Biology and Phylogeny of Snakes. New Hampshire, USA: Science Publishers. pp. 119-181.
24. Spalatin J, Connell R, Burton AN, Gollop BJ. (1964) Western equine encephalitis in saskatchewan reptiles and amphibians, 1961-1963 Can J Comp Med Vet Sci 28(6): 131-142.

25. Ritchie B. (2006) Virology. In: Mader D, editor. Reptile Medicine and Surgery (Second Edition). Saint Louis: W.B. Saunders. pp. 391-417.

26. Muller M, Sachsse W, Zangger N. (1990) Herpesvirus epidemic in greek (testudo hermanni) and maurus land tortoises (testudo graeca) in switzerland Schweiz Arch Tierheilkd 132(4): 199-203.

27. ORIGGI FC. (2006) Reptile medicine and surgery; herpesvirus in tortoises : 814 <last_page> 821.

28. Hyndman TH, Johnson RS. (2015) Evidence for the vertical transmission of sunshine virus Vet Microbiol 175(2-4): 179-184.

29. Langmead B, Salzberg SL. (2012) Fast gapped-read alignment with bowtie 2 Nat Methods 9(4): 357-359.

30. Binder S, Lamp A. (2007) Boa constrictor – die abgottsclange. Münster: Natur und Tier - Verlag. 207 p.

31. Aqrawi T, Stohr AC, Knauf-Witzens T, Krengel A, Heckers KO, et al. (2015) Identification of snake arenaviruses in live boas and pythons in a zoo in germany Tierarztl Prax Ausg K Kleintiere Heimtiere 43(4): 239-247.
32. Korzyukov Y, Hetzel U, Kipar A, Vapalahti O, Hepojoki J. (2016) Generation of anti-boa immunoglobulin antibodies for serodiagnostic applications, and their use to detect anti-reptarenavirus antibodies in boa constrictor PLoS One 11(6): e0158417.

33. Ellenberg P, Linero FN, Scolaro LA. (2007) Superinfection exclusion in BHK-21 cells persistently infected with junin virus J Gen Virol 88(Pt 10): 2730-2739.

34. Baird NL, York J, Nunberg JH. (2012) Arenavirus infection induces discrete cytosolic structures for RNA replication. J Virol 86(20): 11301-11310.

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

36. Mewis JL, Sun X, Zuidhof MJ, Guan LL. (2014) Research note: Methodology for high-quality RNA extraction from poultry whole blood for further gene expression analysis Br Poult Sci 55(2): 194-196.

37. Kallio MA, Tuimala JT, Hupponen T, Klemela P, Gentile M, et al. (2011) Chipster: User-friendly analysis software for microarray and other high-throughput data BMC Genomics 12: 507-2164-12-507.

38. Okonechnikov K, Golosova O, Fursov M, UGENE team. (2012) Unipro UGENE: A unified bioinformatics toolkit Bioinformatics 28(8): 1166-1167.

39. Pickett BE, Sadat EL, Zhang Y, Noronha JM, Squires RB, et al. (2012) ViPR: An open bioinformatics database and analysis resource for virology research Nucleic Acids Res 40(Database issue): D593-8.
40. Thompson JD, Higgins DG, Gibson TJ. (1994) CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22(22): 4673-4680.

41. Abascal F, Zardoya R, Telford MJ. (2010) TranslatorX: Multiple alignment of nucleotide sequences guided by amino acid translations Nucleic Acids Res 38(Web Server issue): W7-13.
**SUPPORTING INFORMATION LEGENDS**

**S1 Table.** Virus names with corresponding abbreviations, GenBank accession numbers, and primer sequences used for L and S segment amplifications by RT-PCR.

**S2 Table.** Coverage and the number of reads matching the *de novo* assembled L and S segments in each NGS sample. Mapping of the raw NGS data was done using Bowtie2.

**S1 Figure.** Raw data of virus species-specific (VSS) RT-PCRs for clutch 1. The RT-PCR products were separated by agarose gel electrophoresis with GelRed (Biotium) nucleic acid stain pre-cast to gels, the bands visualized under UV-light. The VSS RT-PCR products with L segment primers are presented in left-side panels and S segment primer products on right-side panels.

**S2 Figure.** Raw data of virus species-specific (VSS) RT-PCRs for clutch 2. The RT-PCR products were separated by agarose gel electrophoresis with GelRed (Biotium) nucleic acid stain pre-cast to gels, the bands visualized under UV-light. The VSS RT-PCR products with L segment primers are presented in left-side panels and S segment primer products on right-side panels.

**S3 Figure.** Raw data of virus species-specific (VSS) RT-PCRs for clutch 3. The RT-PCR products were separated by agarose gel electrophoresis with GelRed (Biotium) nucleic acid stain pre-cast to gels, the bands visualized under UV-light. The VSS RT-PCR products with L segment primers are presented in left-side panels and S segment primer products on right-side panels.

**S4 Figure.** Raw data of virus species-specific (VSS) RT-PCRs for clutch 4. The RT-PCR products were separated by agarose gel electrophoresis with GelRed (Biotium) nucleic acid stain pre-cast to gels, the bands visualized under UV-light. The VSS RT-PCR products with L segment primers are presented in left-side panels and S segment primer products on right-side panels.
**S5 Figure.** Raw data of virus species-specific (VSS) RT-PCRs for clutch 5. The RT-PCR products were separated by agarose gel electrophoresis with GelRed (Biotium) nucleic acid stain pre-cast to gels, the bands visualized under UV-light. The VSS RT-PCR products with L segment primers are presented in left-side panels and S segment primer products on right-side panels.
Table 1. Clutches, animals and tests performed on individual animals.

A. Summary of results obtained for each animal.

| Clutches, Breeder 1 | Animals (age) | Blood Cytology | PM Histology | Cell Culture | Nucleic acid analysis |
|---------------------|---------------|----------------|--------------|--------------|-----------------------|
| Mother              | Pos           | Pos<sup>B</sup> A [1-7] |             | Pos [brain]  | Brain                 |
| E1.1                |               | Pos<sup>A</sup> A [1, 3, 4, 6] |             | Pos [head]   | Head                  |
| E1.2                |               | Pos<sup>A</sup> A [1, 3, 4, 6] | Neg [body]  |             | Body                  |
| E1.3                |               | Pos<sup>A</sup> A [1, 3, 4, 6] |             |             |                       |
| E1.4                |               | Pos<sup>A</sup> A [1, 3, 4, 6] |             |             |                       |
| E1.5                |               | Pos<sup>A</sup> A [1, 3, 4, 6] |             |             |                       |
| E1.6                | Neg<sup>B</sup> | Pos [CCS] |             |             | CCS                   |
| E1.7                | Pos<sup>B</sup> | Pos [CCS] |             |             | CCS                   |

| Clutches, Breeder 2 | Animals (age) | Blood Cytology | PM Histology | Cell Culture | Nucleic acid analysis |
|---------------------|---------------|----------------|--------------|--------------|-----------------------|
| Mother              | Neg           | Pos<sup>B</sup> A [1-7] |             |             | Brain                 |
| Father<sup>c</sup>  | Neg           |               |             |             |                       |
| E2.1                |               | Pos<sup>B</sup> |             | Pos [CCS]   | Placenta, CC          |
| E2.2                |               | Pos<sup>B</sup> |             | Pos [CCS]   | Body, CC              |
| E2.3                |               | Pos<sup>A</sup> A [1, 3, 4] |             |             | Body                  |

| Clutches, Breeder 1 | Animals (age) | Blood Cytology | PM Histology | Cell Culture | Nucleic acid analysis |
|---------------------|---------------|----------------|--------------|--------------|-----------------------|
| Mother<sup>c</sup>  | Pos           |               |             | Pos [blood]  | Serum                 |
| PNA3.1              | Neg<sup>IB,A</sup> |             |             | Pos [brain]  | Brain                 |
| PNA3.2              | Pos<sup>IB,A</sup> A [1, 4] |             |             | Pos [brain]  | Brain                 |
| PNA3.3              | Neg<sup>IB,A</sup> |             |             | Neg [brain]  | Brain                 |
| J3.1 (2 mo)         | Pos<sup>IB,A</sup> A [1, 2, 4-7] |             |             | Pos [brain]  |                       |
| J3.2 (2 mo)         | Neg<sup>IB,A</sup> |             |             | Pos [brain]  |                       |

| Clutches, Breeder 1 | Animals (age) | Blood Cytology | PM Histology | Cell Culture | Nucleic acid analysis |
|---------------------|---------------|----------------|--------------|--------------|-----------------------|
| Mother<sup>c</sup>  | Neg           | Pos<sup>IB,A</sup> A [1-8] |             | Pos [blood]  | Serum, lung           |
| Father              | Neg           | Pos<sup>IB,A</sup> A [1-8] |             | Pos [blood]  |                       |
| PNA4.1              | Neg<sup>IB,A</sup> |             |             | Pos [brain]  | Placenta, organs      |
| PNA4.2              | Neg<sup>IB,A</sup> |             |             | Pos [lung]   |                       |

| Clutches, Breeder 1 | Animals (age) | Blood Cytology | PM Histology | Cell Culture | Nucleic acid analysis |
|---------------------|---------------|----------------|--------------|--------------|-----------------------|
| Father              | Pos           | Pos<sup>IB,A</sup> A [1, 2, 4-7] |             | Pos [brain]  |                       |
| J5.1 (8 mo)         | Pos           | Pos<sup>IB,A</sup> A [1-7] |             | Neg [blood]  |                       |
| J5.2 (8 mo)         | Pos           | Pos<sup>IB,A</sup> A [1-8] |             | Neg [blood]  |                       |
| J5.3 (8 mo)         | Pos           | Pos<sup>IB,A</sup> A [1-8] |             | Neg [blood]  |                       |
| J5.4 (8 mo)         | Pos<sup>IB,A</sup> A [1-8] |             |             | Pos [blood]  |                       |
| J5.5 (8 mo)         | Neg<sup>IB,A</sup> |             |             | Neg [blood]  |                       |
| J5.6 (8 mo)         | Pos<sup>IB,A</sup> A [1-6, 8] |             |             | Pos [blood]  |                       |
| J5.7 (8 mo)         | Pos<sup>IB,A</sup> A [1-2, 4-8] |             |             | Pos [blood]  |                       |
| J5.8 (12 mo)        | Neg<sup>IB</sup>/pos<sup>A</sup> |             |             | Pos [brain]  |                       |
| J5.9 (12 mo)        | Pos           | Pos<sup>IB,A</sup> A [1-8] |             | Pos [brain]  |                       |
| J5.10 (12 mo)       | Pos           | Pos<sup>IB,A</sup> A [1-8] |             | Pos [brain]  |                       |
| J5.11 (12 mo) | Neg | Neg<sup>IB/(pos)</sup><sup>↑</sup> | Pos [brain] |
| J5.12 (12 mo) | Pos | Pos<sup>IB, A [1-8]</sup> | Pos [brain] |
| J5.13 (12 mo) | Pos | Pos<sup>IB, A [1-8]</sup> | Pos [brain] |
| J5.14 (12 mo) | Pos | Pos<sup>IB, A [1-3, 5-8]</sup> | Pos [brain] |
| J5.15 (12 mo) | Pos | Pos<sup>IB, A [1-8]</sup> | Pos [brain] |
| J5.16 (12 mo) | Pos | Pos<sup>IB, A [1, 3-8]</sup> | Pos [brain] |
| J5.17 (12 mo) | Pos | Pos<sup>IB, A [1, 3-8]</sup> | Pos [brain] |
| J5.18 (12 mo) | Pos | Pos<sup>IB, A [1-8]</sup> | Pos [brain] |
| J5.19 (18 mo) | Pos<sup>IB, A [1-8]</sup> | Pos [brain] |
| J5.20 (18 mo) | Pos<sup>IB, A [1, 3-8]</sup> | Pos [brain] |
| J5.21 (20 mo) | Pos | Pos<sup>IB, A [1-8]</sup> | Pos [blood] |
| J5.22 (20 mo) | Pos | Pos<sup>IB, A [1-8]</sup> | Pos [blood] |

IB - inclusion bodies (as seen in HE stained tissue section or in May Grünwald-Giemsa stained blood smear), PM - post mortem, E - embryo, PNA - perinatal abortion, J - juvenile; CC - cell culture for virus isolation; CCS - supernatant from CC; RT-PCR - reverse transcriptase polymerase chain reaction, NGS - next-generation sequencing; Pos – positive; (pos) – questionable positive; Neg – negative; blank box – not available/not examined; A – tested in tissues by immunohistology; B - tested on formalin-fixed, paraffin-embedded tissue culture pellets by immunocytology; C - Animal still alive. Tissues tested positive by immunohistology: 1 – brain, 2 – heart, 3 – lung, 4 – liver, 5 – pancreas, 6 – kidney, 7 – spleen, 8 – spinal cord.
B. Detailed results obtained from the different specimens used for the RT-PCR approach to identify L segments (abbreviations and accession codes are given in supplementary Table 1, and in materials and methods) in each animal in clutches 1-5. The virus genomes de novo assembled from NGS data of the parental sample and confirmed by VSS RT-PCR indicated by virus abbreviations in bold, and viruses detected by VSS RT-PCR are shown in italics in parents and with + sign in the offspring. +/- sign indicates an ambiguous RT-PCR result.

| Animals          | Specimen     | ABV | SVaV | TSMV | UHV-1/4 | KePV-1 | BSV-1 | HKV | KaBV-1 |
|------------------|--------------|-----|------|------|---------|--------|-------|-----|--------|
| **Clutch 1, Breeder 1** |              |     |      |      |         |        |       |     |        |
| Mother           | Brain        |     |      |      |         |        |       |     |        |
| E1.1             | Head         | +   | +    | +    | +       | +      | +     | +   | -      |
| E1.2             | Body         | -   | -    | +/-  | +       | +      | -     | -   | -      |
| E1.6             | CC (brain)   | -   | -    | +/-  | +       | -      | -     | -   | -      |
| E1.7             | CC (brain)   | -   | -    | +    | +       | +/-   |        |     |        |
| **Clutch 2, Breeder 2** |              |     |      |      |         |        |       |     |        |
| Mother           | Brain        |     |      |      |         |        |       |     |        |
| Father           | Blood        | +/- | -    | -    |         |        |       | -   | -      |
| E2.1             | Salpinx      | +   | +    | +    | +       | +      |        | +/- |        |
|                  | CC (umbilicus)| +   | +    | -    |         | +      |       |     |        |
|                  | CC (kidney)  | -   | -    | -    |         |        |       |     |        |
|                  | CC (heart)   | +   | -    | -    |         |        |       |     | +/-    |
| E2.2             | Body         | +/- | +    | +    | +       | +      |       |     |        |
|                  | CC (umbilicus)| +   | +    | +    | +       | +      |       |     |        |
|                  | CC (placenta)| +   | +    | -    |         | +      |       |     |        |
|                  | CC (kidney)  | -   | +    | -    |         | +      |       |     |        |
|                  | CC (liver)   | +   | +    | -    |         | -      |       |     |        |
| E2.3             | Body         | +/- | +    | +    |         | +      | +/-   |     |        |
| Clutch 3, Breeder 1 | Blood | ABV | SVaV | TSMV | UHV-1/4 | KePV-1 | GMV-1 | KMHV-1 |
|---------------------|-------|-----|------|------|---------|--------|-------|--------|
| Mother              |       |     |      |      |         |        |       |        |
| PNA3.1              |       |     |      |      |         |        |       |        |
| Brain               | -     | -   | -    | -    | -       | -      | -     | -      |
| Kidney              | +/-   | -   | -    | -    | +       | -      | -     | -      |
| Liver               | +     | -   | -    | -    | -       | +      | +/-   | +/-    |
| Brain               | +     | -   | -    | -    | +       | -      | -     | -      |
| Kidney              | +     | -   | -    | -    | -       | +      | +/-   | +/-    |
| Liver               | +/-   | -   | -    | -    | +       | -      | -     | -      |
| PNA3.2              |       |     |      |      |         |        |       |        |
| Brain               | +     | -   | -    | -    | -       | -      | -     | -      |
| Kidney              | +     | -   | -    | -    | +       | +      | +     | -      |
| Liver               | +/-   | -   | -    | -    | -       | +      | +/-   | +/-    |
| PNA3.3              |       |     |      |      |         |        |       |        |
| Brain               | +     | -   | -    | -    | +       | -      | +     | -      |
| Kidney              | +     | -   | -    | -    | -       | +      | +     | +      |
| Liver               | -     | -   | +    | -    | -       | +      | -     | -      |
| J3.1 (2 mo)         |       |     |      |      |         |        |       |        |
| Liver               | -     | -   | +    | -    | -       | +      | -     | -      |
| Kidney              | -     | -   | +    | -    | -       | +      | -     | -      |
| J3.2 (2 mo)         |       |     |      |      |         |        |       |        |
| Liver               | -     | -   | -    | -    | -       | -      | -     | -      |
| Kidney              | -     | -   | +    | -    | +       | -      | +     | -      |

| Clutch 4, Breeder 1 | Blood | ABV-3 | SVaV | TSMV-2 | UHV-1/4 | BSV-1 | GMV-1 | KMHV-1 |
|---------------------|-------|-------|------|--------|---------|-------|-------|--------|
| Father              |       |       |      |        |         |       |       |        |
| Brain               | -     | -     | -    | TSMV-2 | -       | -     | -     | -      |
| Lung                | +/-   | +     | -    | +      | +       | +     | +     | +/-    |
| Mother              |       |       |      |        |         |       |       |        |
| Serum               | +/-   | +     | -    | -      | +       | -     | -     | -      |
| PNA4.1              |       |       |      |        |         |       |       |        |
| Placenta            | +/-   | +     | +/-  | -      | +       | -     | +     | +/-    |
| Kidney              | +     | +/-   | +/-  | +      | +       | +     | +     | +      |
| Lung                | +     | +/-   | +/-  | +      | +       | -     | +     | +/-    |
| PNA4.2              |       |       |      |        |         |       |       |        |
| Placenta            | +     | -     | +/=/  | -      | +       | +     | +     | +/-    |
| Kidney              | +     | -     | +/=/  | +      | +       | -     | +     | +/-    |
| Lung                | +     | -     | +/=/  | -      | +       | -     | -     | +      |
| Brain               | +/-   | +/-   | +/-  | +/=/   | -       | -     | -     | +      |

| Clutch 5, Breeder 1 | Blood | ABV-3 | SVaV | TSMV-2 | UHV-1/4 | BSV-1 | GMV-1 | KMHV-1 |
|---------------------|-------|-------|------|--------|---------|-------|-------|--------|
|                      |       |       |      |        |         |       |       |        |
| Father       | Blood | \(ABV\) | \(SV_{40V}\) | \(TSMV\) - 2 | \(UHV\) - 1/4 | \(KePV\) - 1 |
|--------------|-------|---------|-------------|--------------|---------------|---------------|
| J5.8 (12 mo) | Liver | +       | +           | +/-          | +/-           | +             |
| J5.8 (12 mo) | Brain | +       | -           | -            | -             | +             |
| J5.9 (12 mo) | Liver | +/-     | -           | -            | -             | -             |
| J5.10 (12 mo)| Liver | +       | -           | +/-          | +             | -             |
| J5.11 (12 mo)| Liver | +       | +           | +            | +             | +             |
| J5.12 (12 mo)| Liver | +       | +           | +            | +             | +             |
| J5.13 (12 mo)| Liver | +       | +           | +            | -             | +             |
| J5.14 (12 mo)| Liver | +       | -           | -            | -             | +             |
| J5.15 (12 mo)| Brain | +       | +           | +            | +             | +             |
| J5.18 (12 mo)| Liver | +       | -           | -            | -             | -             |

Bold – L segments derived by NGS and de novo assembly, blank box – not tested.
C. Detailed results obtained from the different specimens used for the RT-PCR approach to identify S segments (abbreviations and accession codes are given in supplementary Table 1, and in materials and methods) in each animal in clutches 1-5. The virus genomes de novo assembled from NGS data of the parental sample and confirmed by VSS RT-PCR are indicated by virus abbreviations in bold, and viruses detected by VSS RT-PCR are shown in italics in parents and with + sign in the offspring. +/- sign indicates an ambiguous RT-PCR result.

| Animals               | Specimen   | Viruses detected by virus species-specific (VSS) RT-PCR |
|-----------------------|------------|--------------------------------------------------------|
|                       |            | S5-like | UGV-1 & -4 | ABV-2 | UGV-2 & -3 | TSMV-2 |
| **Clutch 1, Breeder 1** |            |         |            |        |            |        |
| Mother                | Brain      | S5-like | +           | +      |            |        |
| E1.1                  | Head       |         |             |        |            |        |
| E1.2                  | Body       | +        | -           |        |            |        |
| E1.6                  | CC (brain) | -        | -           |        |            |        |
| E1.7                  | CC (brain) | -        | +           |        |            |        |
| **Clutch 2, Breeder 2** |            |         |            |        |            |        |
| Mother                | Brain      |         | UGV-1 & -4  |        | ABV-2      |        |
| Father                | Blood      |         | -           | -      |            |        |
| Salpinx               | Salpinx    | +        | +           |        |            |        |
| E2.1                  | CC (umbilicus) | -      | +           |        |            |        |
|                       | CC (kidney) | +        | -           |        |            |        |
|                       | CC (heart)  | -        | +           |        |            |        |
| E2.2                  | Body       | -        | -           |        |            |        |
|                       | CC (umbilicus) | +      | +           |        |            |        |
|                       | CC (placenta) | -    | -           |        |            |        |
|                       | CC (kidney) | +        | +           |        |            |        |
|                       | CC (liver)  | +        | +           |        |            |        |
| E2.3                  | Body       | -        | -           |        |            |        |
| **Clutch 3, Breeder 1** |            |         |            |        |            |        |
| Mother                | Blood      | S5-like |            |        | UGV-2 & -3 |        |
|        | Brain | Kidney | Liver |         |         |
|--------|-------|--------|-------|---------|---------|
| PNA3.1 | +     | +      |       | +       |         |
| PNA3.2 |       | +      | +     |         |         |
| PNA3.3 |       | +      |       | +       |         |
| J3.1 (2 mo) |       | +      |       |         |         |
| J3.2 (2 mo) |       | -      |       |         |         |

**Clutch 4, Breeder 1**

|        | Blood | UGV-2 & -3 | TSMV-2 |
|--------|-------|-------------|--------|
| Father | Blood | NA          | NA     |
|        | Brain |             |        |
|        | Lung  | -           | +      |
| Mother | Serum | -           | -      |
| PNA4.1 | Placenta | -        | +      |
|        | Kidney | -          | +      |
|        | Lung   | -          | +      |
| PNA4.2 | Placenta | +    | +      |
|        | Kidney | -          | +      |
|        | Lung   | -          | +      |
|        | Brain  | -          | +      |

**Clutch 5, Breeder 1**

|        | Liver | S5-like | UGV-2 & -3 |
|--------|-------|---------|-------------|
| J5.8 (12 mo) | Brain | +     | -           |
FIGURE LEGENDS

Figure 1. Confirmation of BIBD in parental animals and offspring. **A, B.** Clutch 1, BIBD-positive mother. **A.** The characteristic cytoplasmic inclusion bodies (IB; arrows) are present in erythrocytes (left, blood smear, May-Grünwald Giemsa stain) and in cells in tissues (brain). **B.** Immunohistology confirms the presence of abundant reptarenavirus antigen in all cell types in association with the presence of the IB. **C.** Clutch 2, embryo (E2.1). A few neurons in the spinal cord exhibit reptarenaviral antigen in the cytoplasm (arrowheads). **D.** Clutch 2, embryo (E2.1). Cell pellet from a brain cell culture. Left: passage 1, right: passage 6. There are individual cells expressing viral antigen (arrowheads). **E, F.** Clutch 3, perinatal abortion (PNA2.2), brain. **E.** A few individual neurons exhibit BIBD IBs (arrows). **F.** Reptarenavirus antigen expression is seen in association with inclusion bodies and dispersed in the cytoplasm (arrows). **G, H.** Clutch 5, juvenile (J5.4), 8 mo, brain. **G.** Abundant IB (arrows) are seen within almost all cells. **H.** Reptarenavirus antigen expression is seen in association with inclusion bodies. **A, E, G:** haematoxylin eosin stain; **B, D, F, H:** HRP method, haematoxylin counterstain. Bars = 10 µm.

Figure 2. Evolutionary relationships of the reptarenaviruses sequenced in this study. **A) A** Maximum-likelihood tree built on RdRp nt sequences or **B) complete S segment nt** sequences. For simplicity only a single representative of each L or S segment identified by Stenglein et al. is shown. The abbreviations and accession codes are listed in materials and methods. Bootstrap support values are shown at the nodes.

Figure 3. Vertical transmission of viruses presented in the form of a pedigree for the clutches with embryos and perinatal abortions. The viruses sequenced by NGS are indicated by different colours, no samples were available for the fathers of clutches 1 and 3 (indicated by crossed empty box).
Figure 3