A cluster of Chlamydia serpentis cases in captive snakes

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Abstract

Following the occurrence of sudden death cases in a zoo reptile collection, histological analyses conducted on tissues from two common adders suggested an infection due to *Chlamydia*. The survey was extended to 22 individual snakes from the same collection and a PCR analysis targeting a conserved gene in *Chlamydiaceae* revealed bacterial shedding in six of them. The infection resolved spontaneously in one snake whereas another one succumbed one month later. The antibiotic treatment administered (marbofloxacin) to the remaining four PCR positive animals stopped the mortalities and the shedding. Analysis of the 16S and 23S ribosomal gene sequences

identified *C. serpentis*, a recently described novel chlamydial species in snakes. A PCR tool for a quick and specific identification of this new chlamydial species was developed in this study.

Highlights

- *C. serpentis* cases in captive snakes
- efficient antibiotic treatment with Marbofloxacin
- *C. serpentis* specific real-time PCR

Short Communication

Chlamydiae are obligate intracellular bacteria that cause a variety of human and animal diseases (Rohde et al., 2010). *Chlamydia (C.) serpentis*, *C. poikilothermis*, as well as Candidati *C. corallus*, and *C. sanzinia* are newly discovered or proposed chlamydial species identified so far in captive snakes (Taylor-Brown et al., 2016, 2017; Staub et al., 2018). Very little knowledge is available about their pathogenic potential. Here, we report a cluster of *C. serpentis* cases among captive snakes, along with the therapeutic protocol adopted and the new PCR-based detection tool developed for a quick and specific identification of this new chlamydial species.

In the Parc Zoologique de Paris, two common adders *(Vipera berus,* female ZB4023 and male ZB4024) kept in racks died in summer 2018, less than a month apart. The 3-years old female ZB4023 initially exhibited stomatitis and suffered from anorexia for two months. A treatment with long acting penicillin (Shotapen®, 3.5mg/kg benzylpenicillin, 4.4mg/kg dihydrostreptomycin, IM, every 3 days, during 12 days) was initiated, but the stomatitis remained. Neurological signs (tilted head) appeared one month later, the animal was treated with metronidazole (Flagyl®, 20mg/kg, PO, once a day during 5 days), but it eventually died. Necropsy was performed and the subsequent histological analysis revealed chronic multifocal hepatitis, splenitis, stomatitis, esophagitis, enteritis, myocarditis, oophoritis and encephalitis with granuloma formation as typically seen in cases of chlamydial infection in snakes (Jacobson et al., 1989). While the male ZB4024 did not

show any clinical sign and seemed to be in good physical condition until the day of death, the necropsy revealed global lung congestion and necrosis in the distal 3-4 cm of the caudal lung parenchyma suggesting a fibrinous and necrotizing pneumonia. The presence of *Chlamydia* in multifocal granulomas in inner organs of these two snakes was confirmed by immunohistochemistry using a *Chlamydiaceae* family specific monoclonal antibody directed against the chlamydial lipopolysaccharide (ACI-P, Progen Biotechnik) (**Figure 1**).

As a follow-up of these two *Chlamydia*-positive cases, 22 individual snakes belonging to five different species from the zoo collection were identified as potential "contact animals" after a risk analysis and then, were sampled for PCR analysis (**Table 1, Figure 2**). Tracheal and cloacal swabs were taken and DNA extraction (Qiamp DNA mini kit, Qiagen, France) was performed followed by a real-time PCR targeting the *Chlamydiaceae* family as previously described (Ehricht et al., 2006). Samples with Ct values higher than 39 were considered negative.

The first set of analysis was performed on eight specimens on August 24th 2018 (**Table 1**). The ZB4025 common adder, housed in a rack close to ZB4023 and ZB4024, tested positive (Ct 26 and 29 for the trachea and cloaca, respectively) as well as three aspics (*Vipera aspis)*, which were hosted three months earlier in racks (**Figure 2**). A viperine snake (*Natrix maura,* ZB8018) kept in proximity of the common adders tested negative. Despite the administration of antibiotic treatment (oxytetracycline 10 mg/kg, every 2 days, five doses), the ZB4025 common adder died few days after the end of treatment. Like ZB4023, this adder exhibited ptyalism and suffered from anorexia and stomatitis. PCR analysis revealed a high level of *Chlamydiaceae* infection in all organs, with highest Ct values obtained for heart (Ct 19.2) and brain (Ct 17.6) tissues, as also confirmed by the histological analysis (presence of chlamydial inclusions, data not shown).

On October 9th, the PCR screening (**Table 1**) was extended to *Natrix maura* (n=5), *Sanzinia madagascariensis* (n=4) and *Rhinechis scalaris* (n=3) specimens kept in the Zoo de Paris collection. The three *Vipera berus* and the three *Vipera aspis* previously tested by PCR on August were re-

tested. Shedding was confirmed for two *Vipera aspis* (ZB7047 and ZB7048) whereas two *Rhinechis scalaris* specimens tested positive (ZB4614 and ZB3104). These four PCR positive animals were treated with marbofloxacin (5 mg/kg, IM, single in day, until PCR results became negative), following a protocol previously reported for infection with *C. pneumoniae* in snakes (Rüegg et al., 2015). The tracheal and cloacal shedding was monitored by PCR each week. Within less than 45 days later, all animals tested negative (**Table 1**). Through clinical exam, no toxicity of the treatment was observed. Interestingly, the *Vipera aspis* ZB7045 tested positive in August was negative by PCR in October despite any treatment administered. Spontaneous resolution of infection without antibiotic administration was previously reported for *C. pneumoniae* (Rüegg et al., 2015).

Further molecular characterization of the chlamydial species circulating in this snake collection was performed. The almost full length 16S (1334 bp) and the partial 23S (922 bp) rRNA gene sequences of ZB4025, ZB7047 and ZB7048 were amplified as previously described (Aaziz et al., 2015). Sequences were all identical and their comparison with sequences present in the databases showed a high sequence identity rate (more than 99.7%) with H15-1957-10C, the reference strain for *C. serpentis*. To note, the 16S rRNA sequences were identical to the genotype 1 (2464-255) sequence obtained from a *Montivipera albizona* specimen (Taylor-Brown et al., 2015).

From the whole genome sequence of *C. serpentis* (Staub et al., 2018), specific primers Serpentis_192F (5‘-TGA AGA CTT AAG AGA AGA TGC GGT-3’) and Serpentis_291R (5‘-TGC GGG GAC TTT TAC TAG CC-3’) as well as a specific probe Serpentis_251P (5’-FAM- ACG TTC CAG AGT CTT TAG GGG-TAMRA 3’) were designed using the primer3 software. This specific qPCR assay was designed based on the Glutamyl-tRNA(Gln) amidotransferase subunit C (*gat*C) gene locus only identified so far in *C. serpentis*. Amplification was performed using the 2X TaqMan Universal PCR Master Mix (Applied Biosystems, Courtaboeuf, France). The final volume of the reaction mixture was 20 µL including 10 µL of master mix, 2 µL of DNA sample, 0.6 µM of each primer, 0.1 µM of the probe and sterile PCR water. Amplification was carried out on a Viia7

thermocycler (Applied Biosystems) using the following cycling parameters: heating at 95 °C for 10 min, 50 cycles of 95 °C for 15 s and 60 °C for 1 min. The specificity of the new real-time PCR system was evaluated *in silico* and validated on 32 *Chlamydiaceae* strains, including the H15-1957-10C reference strain of *C. serpentis* (**Table S1**). All *Chlamydiaceae* PCR-positive samples from this study tested positive with this new PCR system confirming the circulation of *C. serpentis* among these snakes.

The administration of a targeted antibiotic treatment to *Chlamydia*-infected snakes depends on the establishment of a correct diagnosis of chlamydiosis, a disease due to an obligate intracellular bacterium not included in the list of first-line pathogens affecting snakes. This cluster of cases among captive snakes as well as other recent reports (Frutos et al., 2014, Rüegg et al., 2015) showed that chlamydiae do not necessarily induce clinical signs in their hosts, but that the outcome can be fatal if not treated. The chlamydial infection source for snakes remains unknown to date. Interestingly, the three PCR-positive *Vipera aspis* were hosted in the rack storage, where the three *Vipera berus* died. Racks were cleaned and quickly disinfected before the introduction of the new snakes. These animals, without any apparent clinical signs, could have been infected from their new environment incompletely disinfected or healthy carriers, without direct or indirect link with the fatal cases. No other circulating pathogen that could have induced an immunocompromised status was identified in this snake population, but a mite infestation (*Ophionyssus natricis*) was noted 3 weeks before the first death (ZB4024). All three common adders (ZB4023, ZB4024 and ZB4025) were treated with fipronil (Frontline®, topical on skin, once) and the distant environment was fumigated with one insecticide combination (Moxy®: pyrethrin, permethrin, pyperonyl butoxid). While relevance of this ectoparasite presence remains unknown regarding onset of clinical signs, the ability of *Chlamydia* mechanical transmission through such a blood suckling arthropod could not be ruled out (Emmerson et al, 2000).

PCR stands as a powerful tool for the diagnosis of *Chlamydia* infection and shedding in snakes, as chlamydial culture remains a technical challenge and has a low sensitivity, leading to false negative results (Jacobson et al, 1989).

The only species that remained negative among all contact animals were snakes of the species *Sanzinia madagascarensis*. Of note, this is also the snake species with highest preferred body temperature range among contact species. Different incubation temperatures may variably impact replication of individual chlamydial species as recently shown by Onorini et al, 2019. Moreover, the existence of several chlamydial species in snakes may indicate their species-driven specificity for certain ophidian species or genera hosts.

The zoonotic potential of the new species *C. serpentis* is yet unknown but it shows a high phylogenetic relationship to *C. pneumoniae*, which is zoonotic and also detected in herpetofauna (Ebani, 2017). None of the people in contact with the infected snakes reported specific clinical signs concomitantly to the circulation of *C. serpentis* in these snakes, but basic protective measures (gloves) are included in daily husbandry protocols, and were enhanced (mask) during the course of treatment and sampling.

Since these index cases, all snakes entering the collection of the zoo are tested for *Chlamydiaceae* by PCR, as routine quarantine examination.

Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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150 **References**

151 Aaziz R, Gourlay P, Vorimore F, Sachse K, Siarkou V, Laroucau.K. 2015. Occurrence and diversity
152 of *Chlamydiaceae* spp. in North Atlantic seabirds admitted to a wildlife rescue centre in Western
153 France. Applied and Environmental Microbiology. 81(14):4581-90.

154 Ebani VV. 2017. Domestic reptiles as source of zoonotic bacteria: A mini review. Asian Pacific
155 Journal of Tropical Medicine. 10(8), 723–728.

156 Ehricht R, Slickers P, Goellner S, Hotzel H, Sachse K. 2006. Optimized DNA microarray assay
157 allows detection and genotyping of single PCR-amplifiable target copies. Mol Cell Probes.
158 20(1):60-63.

159 Emerson PM, Bailey RL, Mahdi SO, Walraven GEL, Lindsay SW. 2000. Transmission ecology of
160 the fly *Musca sorbens,* a putative vector of trachoma, Transactions of The Royal Society of
161 Tropical Medicine and Hygiene, 94(1) : 28–32

162 Frutos MC, Monetti MS, Ré VE, Cuffini CG. 2014. Molecular evidence of Chlamydophila
163 pneumoniae infection in reptiles in Argentina. Rev Argent Microbiol. 46(1):45-8.

164 Jacobson E, Gaskin J, Mansell J. 1989. Chlamydial Infection in Puff Adders (Bitis arietans). Journal
165 of Zoo and Wildlife Medicine. 20(3), 364-369.

166 Mitchell CM, Hutton S, Myers GS, Brunham R, Timms P. 2010. Chlamydia pneumoniae is
167 genetically diverse in animals and appears to have crossed the host barrier to humans on (at
168 least) two occasions. PLoS Pathog. 20;6(5):e1000903.

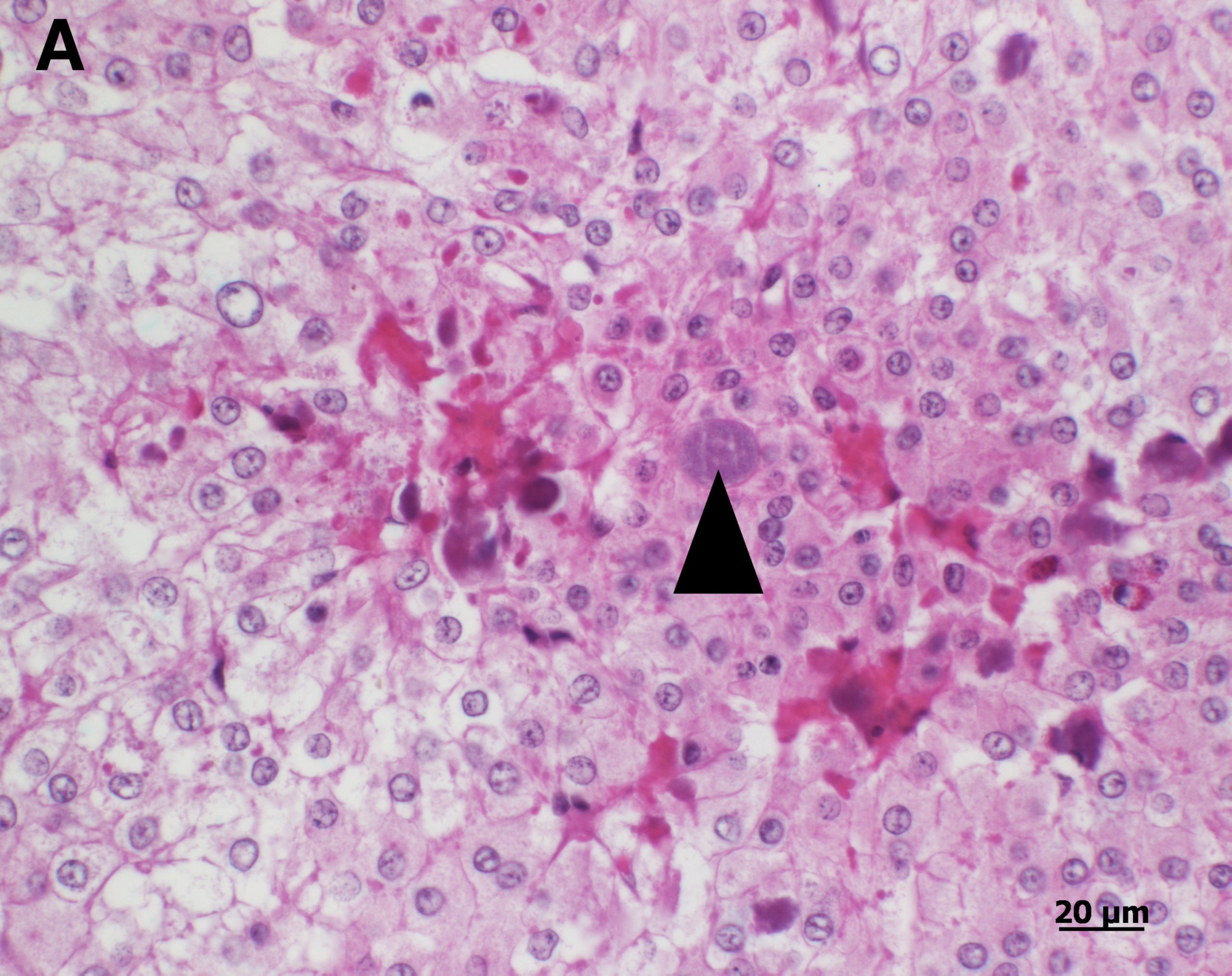
169 Onorini D, Donati M, Marti H, Biondi R, Levi A, Nufer L, Prähauser B, Rigamonti S, Vicari N,
170 Borel N. 2019. The influence of centrifugation and incubation temperatures on various
171 veterinary and human chlamydial species. Veterinary Microbiology, 233, 11–20.

172 Staub E, Marti H, Biondi R, Levi A, Donati M, Leonard CA, Ley SD, Pillonel T, Greub G, Seth-
173 Smith HMB, Borel N. 2018. Novel Chlamydia species isolated from snakes are temperature-
174 sensitive and exhibit decreased susceptibility to azithromycin. Sci Rep. 8(1):5660.
175 Taylor-Brown A, Spang L, Borel N, Polkinghorne A. 2017. Culture-independent metagenomics
176 supports discovery of uncultivable bacteria within the genus Chlamydia. Sci Rep. 7(1):10661.
177 Taylor-Brown A, Bachmann NL, Borel N, Polkinghorne A. 2016. Culture-independent genomic
178 characterisation of Candidatus Chlamydia sanzinia, a novel uncultivated bacterium infecting
179 snakes. BMC Genomics. 17:710.
180 Taylor-Brown A, Rüegg S, Polkinghorne A, Borel N. 2015. Characterisation of Chlamydia
181 pneumoniae and other novel chlamydial infections in captive snakes. Vet Microbiol. 178(1-
182 2):88-93.
183 Rohde G, Straube E, Essig A, Reinhold P, Sachse K. 2010. Chlamydial zoonoses. Dtsch Arztebl Int.
184 107(10):174-80.
185 Rüegg SR, Regenscheit N, Origgi FC, Kaiser C, Borel N. 2015. Detection of Chlamydia
186 pneumoniae in a collection of captive snakes and response to treatment with marbofloxacin. Vet
187 J. 205(3):424-6.
188
189 **Tables & Figures**
190 **Figure 1.** Histopathology (haematoxylin and eosin staining, panel A and C) and
191 immunohistochemistry (3-amino-9-ethylcarbazole (AEC)-peroxidase method with haematoxylin
192 counterstain, panel B and D) for *Chlamydiaceae* antigen of snake ZB4023 (*Vipera berus*) that was
193 positive for *C. serpentis*. Magnification 400x. A. Large histiocytic granuloma in the heart with
194 intra-histiocytic basophilic chlamydial inclusion (arrowhead). B. Positive red granular
195 immunostaining in the cytoplasm of histiocytes within a granuloma of the heart (pericardium). C.
196 Granulomatous oophoritis with intralesional chlamydial inclusions (arrowheads). D. Positive red
197 granular immunostaining in granulomas of the ovary.

Figure S1. Movements of reptiles in May and their location in August 2018.

Table 1. Data summary table

Table S1. Chlamydial strains and isolates used for determination of specificity of the *C. serpentis* specific real-time PCR assay.

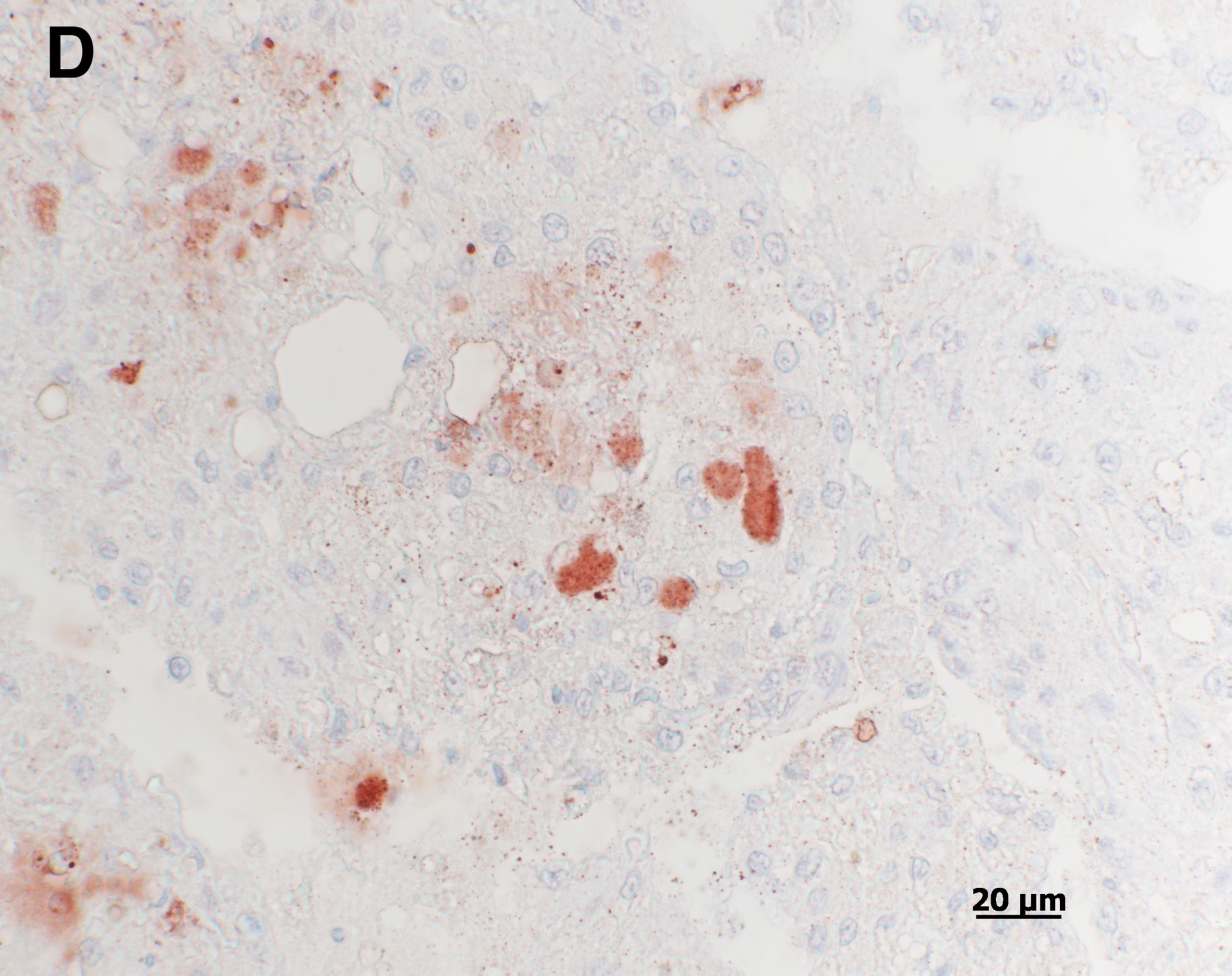

A
20 µm

B

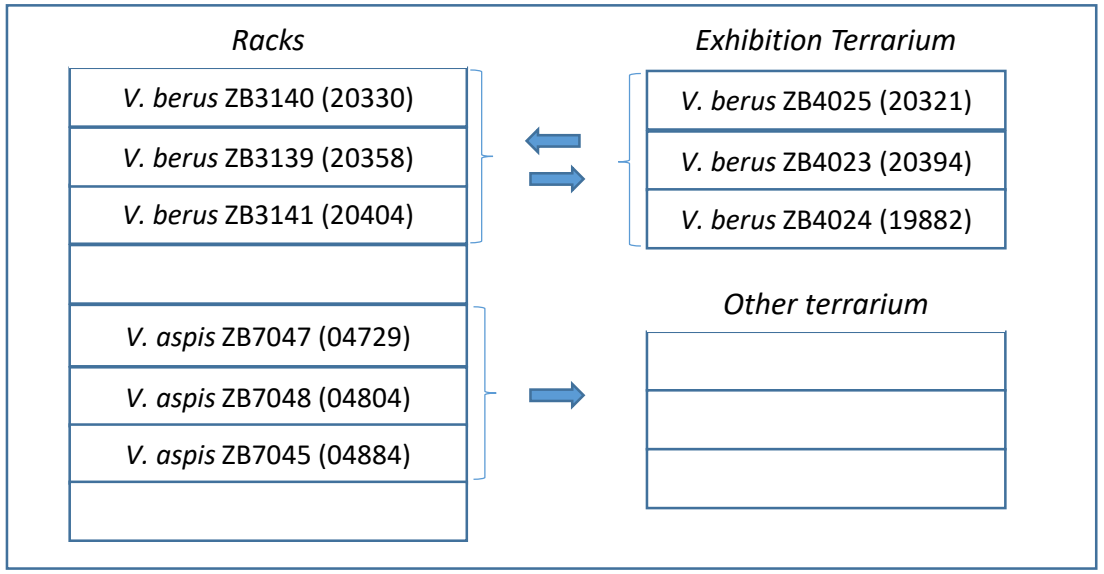
20 µm

C

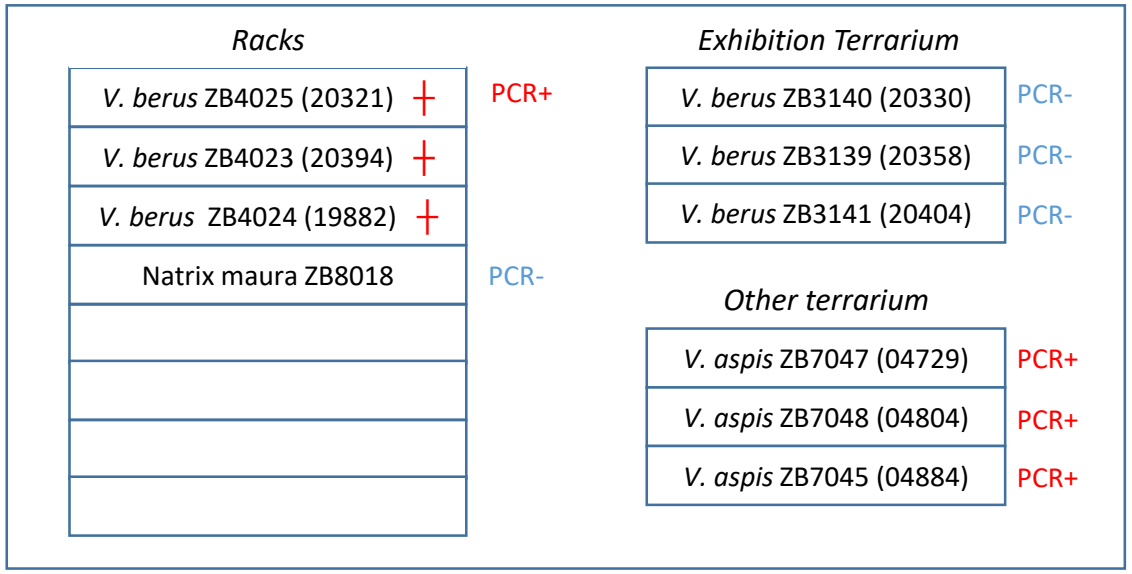
20 µm


D
20 µm

Location in May 2018, before movements



Situation in August 2018



ID	House name ZB	Species	Location after May 2018	PCR *Chlamydiaceae*				Date of death	Histology/Immuno-histochemistry	Antibiotic treatment															
				24/08/2018		09/10/2018					03/11/18		09/11/18		16/11/18		23/11/18		30/11/18		07/12/18		14/12/18	
				Trachea (Ct)	Cloaca (Ct)	Trachea (Ct)	Cloaca (Ct)			started on 29/10/2018	Trachea (Ct)	Cloaca (Ct)	Trachea (Ct)	Cloaca (Ct)	Trachea (Ct)	Cloaca (Ct)	Trachea (Ct)	Cloaca (Ct)	Trachea (Ct)	Cloaca (Ct)	Trachea (Ct)	Cloaca (Ct)	Trachea (Ct)	Cloaca (Ct)
Ec 04729	ZB7047			33.6	29.4	33.7	31.1			+	35.6	-	38.8	36.0	-	36.4	-	-	-	-	nd	nd	-	-
Ec 04804	ZB7048	*Vipera aspis*	Other Terrarium	31.5	30.0	33.9	31.0			+	35.2	33.8	38.8	32.8	37.2	34.9	-	35.1	-	37.9	-	37.1	-	-
Ec 04884	ZB7045			35.4	34.5	-	-																	
Ec 20330	ZB3140			-	-	-	-																	
Ec 20358	ZB3139	*Vipera berus*	Exhibition terrarium	-	-	-	-																	
Ec 20404	ZB3141			-	-	-	-																	
Ec 20394	ZB4023							03/08/18	+															
Ec 19882	ZB4024	*Vipera berus*	Racks					15/07/18	+															
Ec 20321	ZB4025			26.0	29.0			27/09/18	+															
Ec 16806	ZB8018			-	-																			
Ec 01505	ZB8022					-	-																	
Ec 01554	ZB8023	*Natrix maura*				-	-																	
Ec 01460	ZB8021					-	-	05/02/19																
Ec 30109	ZB8026					-	-																	
Ec 30112	ZB8025					-	-																	
	ZB6233					-	-																	
	ZB6234	*Sanzinia madagascarensis*				-	-																	
	ZB6243					-	-																	
	ZB6244					-	-																	
Ec 19971	ZB4614					-	31.8			+	38.5	-	-	35.8	-	35.9	38.4	36.2	-	37.9	-	35.9	-	-
Ec 20797	ZB3104	*Rhinechis scalaris*				-	35.4			+	-	38.3	-	38.7	-	-	-	-	-	-	nd	nd	-	-
Ec 20369	ZB3122					-	-																	

* nd: not done