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► To cite this version:

K. Laroucau, R. Aaziz, A. Lécu, S. Laidebeure, O. Marquis, et al.. A cluster of *Chlamydia serpentis* cases in captive snakes. *Veterinary Microbiology*, 2020, 240, pp.108499 -. 10.1016/j.vetmic.2019.108499 . hal-03488764

HAL Id: hal-03488764

<https://hal.science/hal-03488764>

Submitted on 21 Jul 2022

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1 Vet. Microbiol.

2 **A cluster of *Chlamydia serpentis* cases in captive snakes**

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16

17 **Abstract**

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

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

27 **Highlights**

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

31

32 **Short Communication**

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

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

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

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

62 The first set of analysis was performed on eight specimens on August 24th 2018 (**Table 1**). The
63 ZB4025 common adder, housed in a rack close to ZB4023 and ZB4024, tested positive (Ct 26 and
64 29 for the trachea and cloaca, respectively) as well as three aspics (*Vipera aspis*), which were
65 hosted three months earlier in racks (**Figure 2**). A viperine snake (*Natrix maura*, ZB8018) kept in
66 proximity of the common adders tested negative. Despite the administration of antibiotic treatment
67 (oxytetracycline 10 mg/kg, every 2 days, five doses), the ZB4025 common adder died few days
68 after the end of treatment. Like ZB4023, this adder exhibited ptyalism and suffered from anorexia
69 and stomatitis. PCR analysis revealed a high level of *Chlamydiaceae* infection in all organs, with
70 highest Ct values obtained for heart (Ct 19.2) and brain (Ct 17.6) tissues, as also confirmed by the
71 histological analysis (presence of chlamydial inclusions, data not shown).

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

75 tested. Shedding was confirmed for two *Vipera aspis* (ZB7047 and ZB7048) whereas two *Rhinechis*
76 *scalaris* specimens tested positive (ZB4614 and ZB3104). These four PCR positive animals were
77 treated with marbofloxacin (5 mg/kg, IM, single in day, until PCR results became negative),
78 following a protocol previously reported for infection with *C. pneumoniae* in snakes (Rüegg et al.,
79 2015). The tracheal and cloacal shedding was monitored by PCR each week. Within less than 45
80 days later, all animals tested negative (**Table 1**). Through clinical exam, no toxicity of the treatment
81 was observed. Interestingly, the *Vipera aspis* ZB7045 tested positive in August was negative by
82 PCR in October despite any treatment administered. Spontaneous resolution of infection without
83 antibiotic administration was previously reported for *C. pneumoniae* (Rüegg et al., 2015).

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

91 From the whole genome sequence of *C. serpentis* (Staub et al., 2018), specific primers
92 Serpentis_192F (5'-TGA AGA CTT AAG AGA AGA TGC GGT-3') and Serpentis_291R (5'-TGC
93 GGG GAC TTT TAC TAG CC-3') as well as a specific probe Serpentis_251P (5'-FAM- ACG
94 TTC CAG AGT CTT TAG GGG-TAMRA 3') were designed using the primer3 software. This
95 specific qPCR assay was designed based on the Glutamyl-tRNA(Gln) amidotransferase subunit C
96 (*gatC*) gene locus only identified so far in *C. serpentis*. Amplification was performed using the 2X
97 TaqMan Universal PCR Master Mix (Applied Biosystems, Courtaboeuf, France). The final volume
98 of the reaction mixture was 20 µL including 10 µL of master mix, 2 µL of DNA sample, 0.6 µM of
99 each primer, 0.1 µM of the probe and sterile PCR water. Amplification was carried out on a Vii7

100 thermocycler (Applied Biosystems) using the following cycling parameters: heating at 95 °C for 10
101 min, 50 cycles of 95 °C for 15 s and 60 °C for 1 min. The specificity of the new real-time PCR
102 system was evaluated *in silico* and validated on 32 *Chlamydiaceae* strains, including the H15-1957-
103 10C reference strain of *C. serpentis* (**Table S1**). All *Chlamydiaceae* PCR-positive samples from this
104 study tested positive with this new PCR system confirming the circulation of *C. serpentis* among
105 these snakes.

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

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

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

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

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

141

142 **Conflict of interest statement**

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

145

146 **Acknowledgement**

147 We are grateful to Dr. Alexandra Nicolier and Dr Karin Lemberger (VetDiagnostics, France) for
148 providing histological blocs.

149

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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.

198

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

200

201 **Table 1.** Data summary table

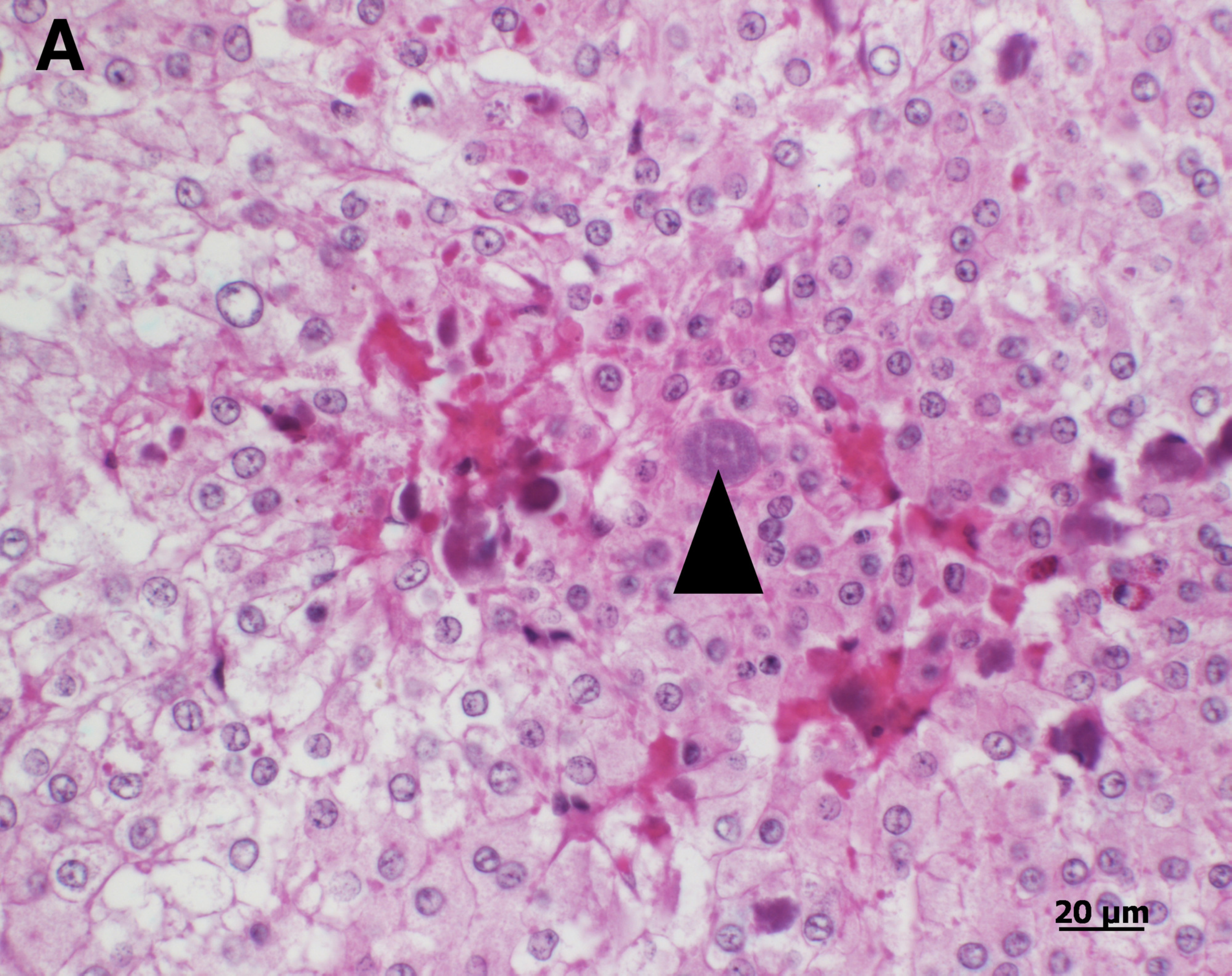
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203 **Table S1.** Chlamydial strains and isolates used for determination of specificity of the *C. serpentis*

204 specific real-time PCR assay.

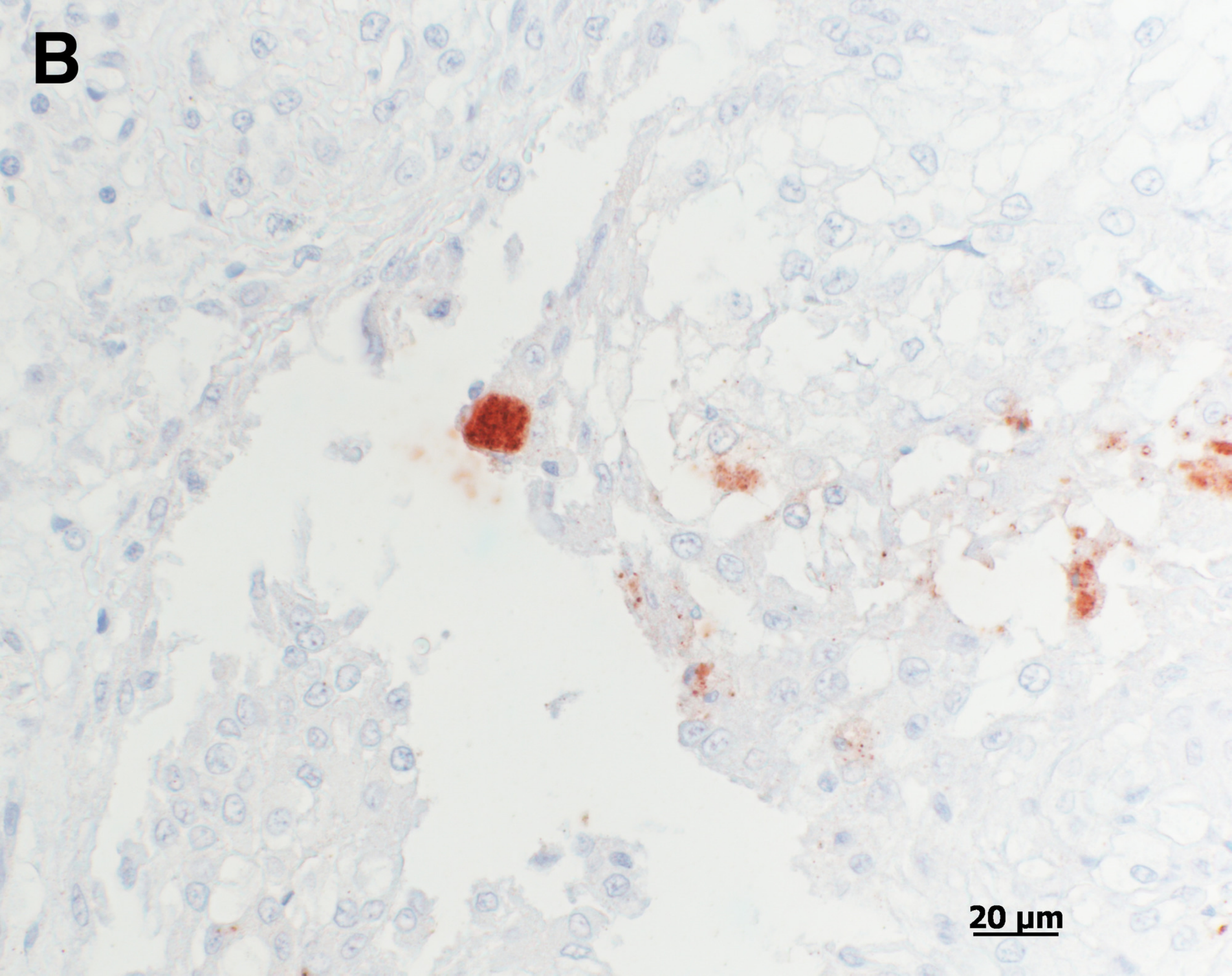
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A



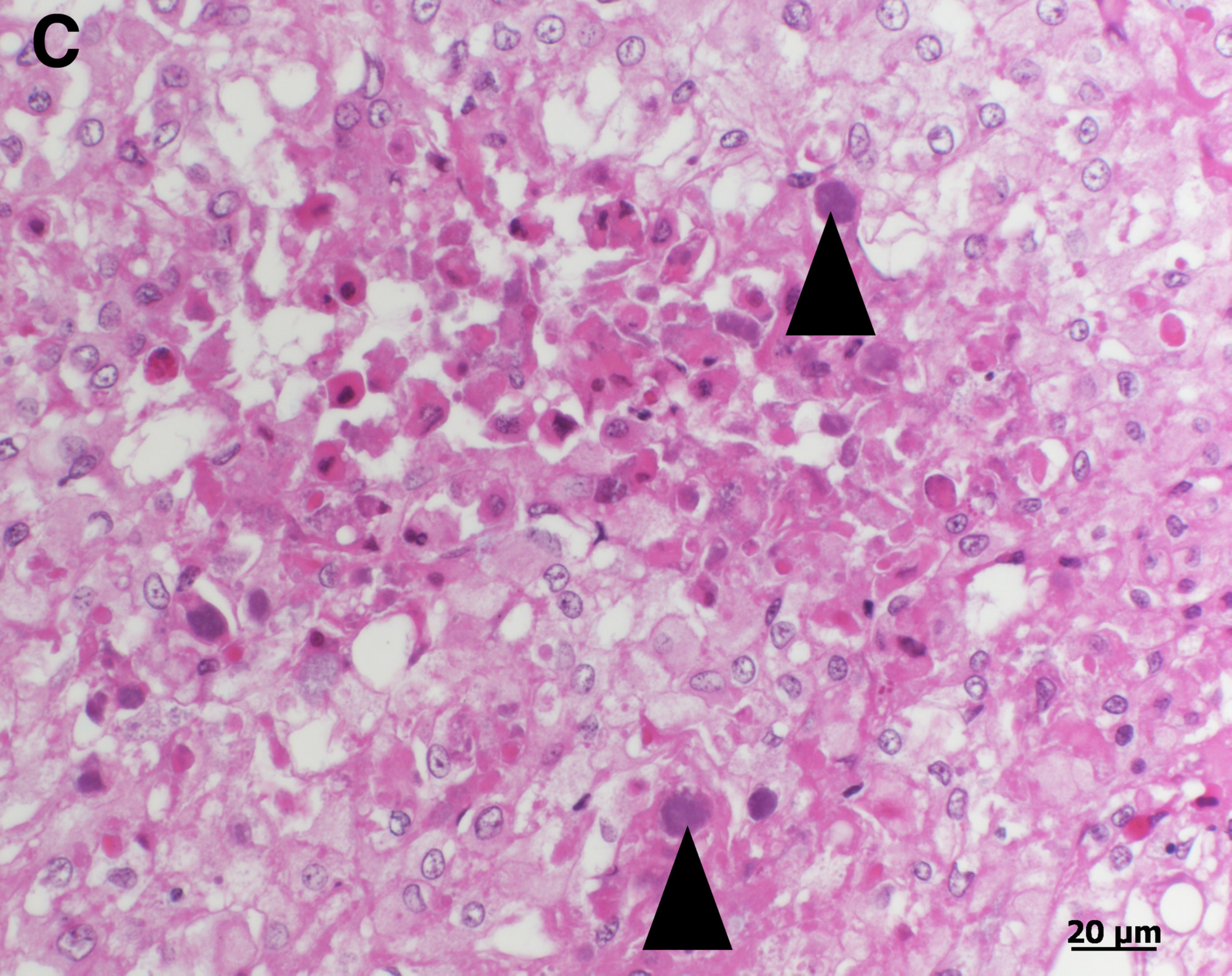
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B



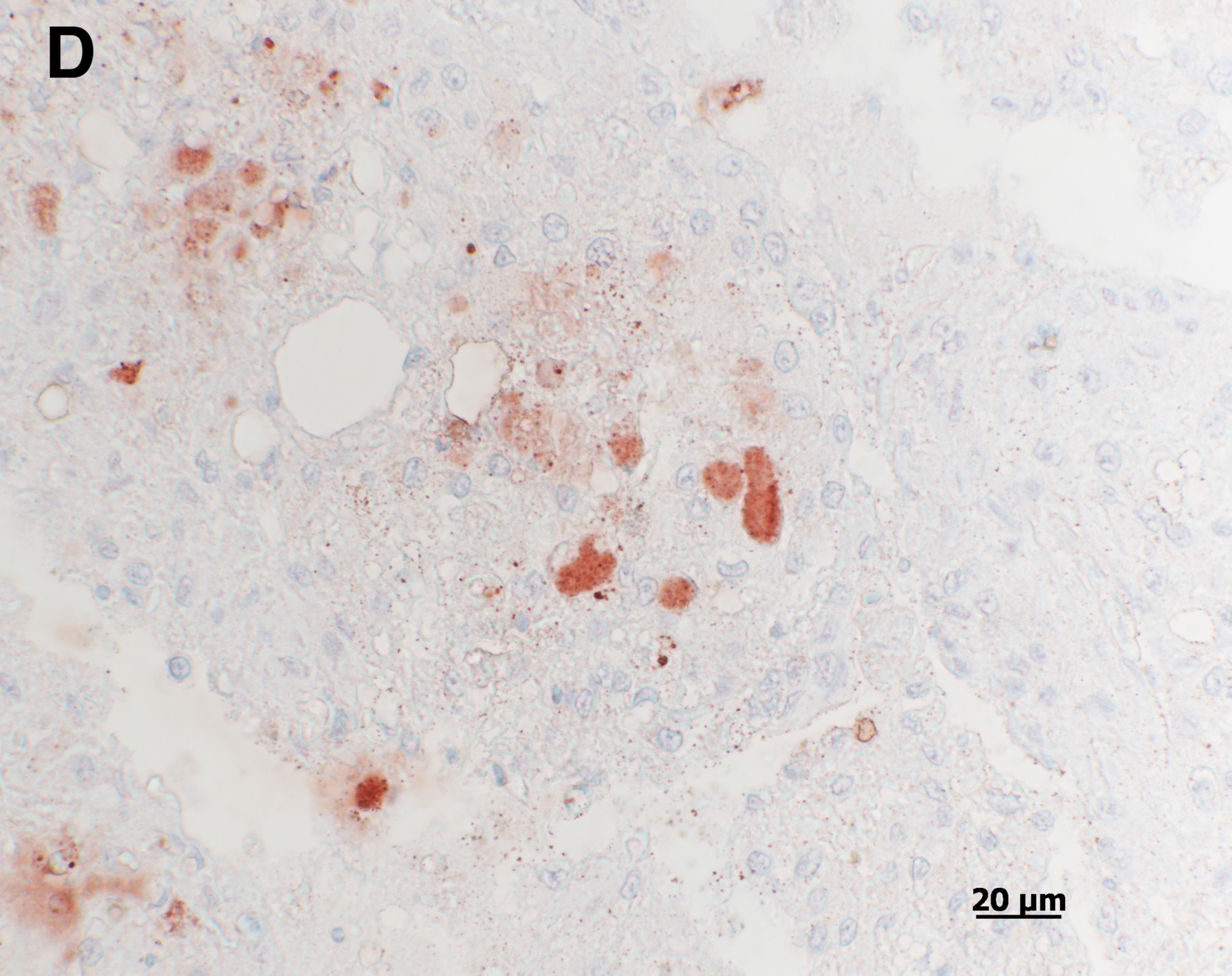
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C



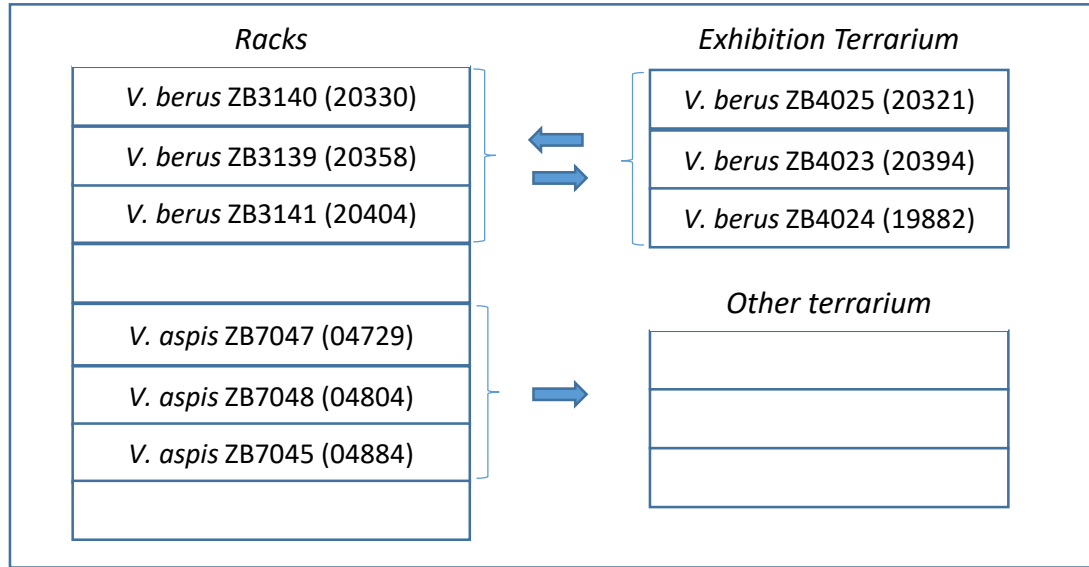
20 μ m

D

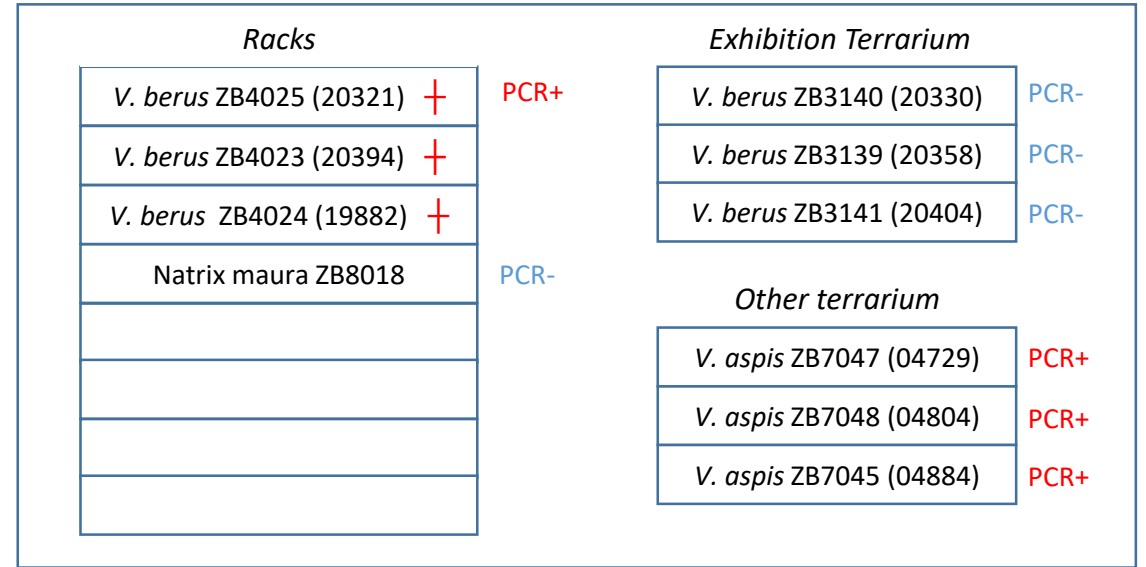


20 μ m

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/Immunohistochemistry	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)			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	<i>Vipera aspis</i>	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	<i>Vipera berus</i>	Exhibition terrarium	-	-	-	-																	
Ec 20404	ZB3141			-	-	-	-																	
Ec 20394	ZB4023							03/08/18	+															
Ec 19882	ZB4024	<i>Vipera berus</i>	Racks					15/07/18	+															
Ec 20321	ZB4025			26.0	29.0			27/09/18	+															
Ec 16806	ZB8018			-	-	-	-																	
Ec 01505	ZB8022					-	-																	
Ec 01554	ZB8023					-	-																	
Ec 01460	ZB8021	<i>Natrix maura</i>				-	-	05/02/19																
Ec 30109	ZB8026					-	-																	
Ec 30112	ZB8025					-	-																	
	ZB6233					-	-																	
	ZB6234	<i>Sanzinia</i>				-	-																	
	ZB6243	<i>madagascarensis</i>				-	-																	
	ZB6244					-	-																	
Ec 19971	ZB4614					-	31.8			+	38.5	-	-	35.8	-	35.9	38.4	36.2	-	37.9	-	35.9	-	-
Ec 20797	ZB3104	<i>Rhinechis scalaris</i>				-	35.4			+	-	38.3	-	38.7	-	-	-	-	-	-	nd	nd	-	-
Ec 20369	ZB3122					-	-																	

* nd: not done

