1 First detection and molecular identification of the zoonotic Anaplasma capra in

2 deer in France

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- 4 Short title : Anaplasma capra in deer in France
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13 Abstract

14 Cervids are known to be reservoir of zoonotic tick-transmitted bacteria. The aim of this study was to perform a survey in a wild fauna reserve to characterize Anaplasma species carried by captive red 15 16 deer and swamp deer. Blood from 59 red deer and 7 swamp deer was collected and analyzed over a 17 period of two years. A semi-nested PCR that targets the 23S rRNA was performed to detect and 18 characterise Anaplasma spp. and determine zoonotic species presence. Anaplasma phagocytophilum 19 was identified in 14/59 deer (23.7%) but not in swamp deer. Few sequences could not be assigned 20 to any particular species based on the 23S rRNA sequences. Nested PCR targeting 16S rRNA, gltA 21 and groEL genes and sequencing analysis detected a recently reported zoonotic species, Anaplasma 22 *capra* in red deer as well as in swamp deer. This is the first reporting of the tick-borne zoonotic 23 bacterium A. capra in France, a species otherwise described only in China and Japan, in goats, 24 sheep, deer and japanese serows. Even if this bacterium may have been introduced in the Park with 25 infected imported animals, its local epidemiological cycle through tick transmission seems possible 26 as locally born deer were found infected. Diagnostic methods, especially molecular ones, should 27 take into account the potential infection of animals and humans with this species. 28 29 Keywords: Anaplasma capra, zoonotic tick-borne bacteria, red deer, swamp deer, 23S rRNA, 16S 30 rRNA, groEL, gltA

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34 Introduction

35 Bacteria of the genus Anaplasma are obligate intracellular parasites that replicate within the 36 vacuoles of diverse eukaryotic cells (monocytes, granulocytes, erythrocytes, endothelial cells). 37 These bacteria are mainly transmitted by ixodid ticks and multiply both in the invertebrate and 38 vertebrate hosts [1]. The genus Anaplasma includes 6 recognized species (A. phagocytophilum, A. 39 bovis, A. centrale, A. marginale, A. ovis and A. platys) responsible for anaplasmosis worldwide on a 40 large range of wild and domesticated vertebrates [1]. One species, A. phagocytophilum, described in 41 1994 in the USA as the agent of human granulocytic anaplasmosis (HGA), is now increasingly 42 detected worldwide [1]. In 2015, a second zoonotic and new species, proposed as A. capra, was 43 described in humans in China [2]. On a population of 477 patients with tick-bite history, six percent 44 (28 patients) were found infected with A. capra with non-specific febrile manifestations. Five of 45 them were hospitalized due to severe symptoms. General clinical features in human patients have 46 included febrile manifestations (fever, headache, malaise) as well as eschar, lymphadenopathy and 47 gastrointestinal symptoms [2]. 48 Both A. phagocytophilum and A. capra infect diverse domestic (sheep and goats) and wild 49 ruminants (deer) species, which are considered as reservoirs. In a survey of tick-borne diseases in 50 captive and protected deer in France, we investigated the presence of Anaplasma species infecting 51 captive red deer (Cervus elaphus) and swamp deer (Rucervus duvaucelii). In the "Réserve de la 52 Haute Touche", endangered species such as the swamp deer (CITES appendix I) are preserved. This reserve is surrounded by a large forested and humid area, a biotope favorable to Ixodid ticks, 53 54 vectors of A. phagocytophilum.

55 Methods

56 Animal sampling

In 2015, a molecular survey of *Anaplasma* spp. infecting deer was started in the "Réserve de
la Haute Touche" Zoological Park, Indre, France (National Museum of Natural History). Blood

- samples from 59 red deer and 7 swamp deer were collected between 2015 and 2017. They were
- 60 used for molecular detection and characterization of Anaplasma spp.. Blood was sampled at the
- 61 jugular vein at the occasion of animal care (treatments, vaccinations) or transfers (authorization 36-

62 145-002).

- 63 Molecular detection and characterization of Anaplasma spp.
- 64 Genomic DNA was extracted from blood according to previously described protocols [3]. We
- 65 detected Anaplasmatacae by semi-nested PCR based on the 23S rRNA gene [4] and determined the
- 66 species by sequencing PCR positive amplicons. A new detected Anaplasma species was further
- 67 characterized using nested PCR and sequencing of the *16S* rRNA, *gltA* and *groEL* genes (table 1).
- 68 Bidirectionnal sequencing was performed to ensure sequences, that were further analyzed by the
- 69 BLASTN (<u>http://www.ncbi.nlm.nih.gov/BLAST/</u>) and CLUSTAWL
- 70 (<u>https://www.ebi.ac.uk/Tools/msa/clustalo/</u>) programs.
- 71 Results

72 Detection of Anaplasma spp. in deer blood

73 Of the 66 heparine blood samples from red deer and swamp deer, 23S rRNA amplicons of the right

size were obtained for 28 samples. A BLASTn search of 23S rRNA sequences identified A.

- 75 *phagocytophilum* in 14 red deer (4/21 in 2015, 7/23 in 2016 and 3/15 in 2017) (prevalence of
- 76 23.7%) but not in swamp deer. Sequences (lengths between 430-476 bp) were more than 99.5%
- 77 identical (maximum two mismatches) to A. phagocytophilum strain HZ (GenBank accession
- number NR_076399). Sequences from eleven amplicons (367 to 476 bp) were identical, with 99.8%
- 79 identities with Ralstonia pickettii (GenBank accession number CP001644). Three identical
- 80 sequences from two red deer and one swamp deer (3/66 infection rate 4.5%) gave the highest
- 81 identities with "Candidatus Anaplasma mediterraneum" sequence (KY498330), described as a
- 82 potentially new Anaplasma species infecting sheep in Corsica [5] (table 2). Other genetic markers
- 83 often used to identify Anaplasma species were then tested to further characterize this Anaplasma
- 84 species never described in deer.

85 Further molecular characterization of the new Anaplasma spp. from deer in France

86 Two 16S rRNA identical sequences were obtained from red and swamp deer blood samples. They 87 had similarities of more than 99.6% with numerous 16S rRNA Anaplasma capra sequences 88 deposited in GenBank. These sequences were obtained from sheep, goat, human blood and ticks 89 from China, as well as from cattle, sika deer, Japanese serows and ticks from Japan. Sequence 90 similarities with other known Anaplasma 16S rRNA sequences were lower than 99% (table 2). 91 As Anaplasma capra groEL and gltA sequences were also deposited in GenBank, we 92 amplified and sequenced these genes from our deer blood samples to better characterize this new 93 Anaplasma. The three groEL Anaplasma sequences from deer were identical and identity rates 94 ranged from 91.4 to 97.7% with the A. capra groEL sequences from China and Japan available in 95 GenBank (table 2). The similarities with *groEL* sequences from other related *Anaplasma* species (A. 96 centrale, A. marginale, A. platys, A. phagocytophilum and A. ovis) fell under 84%. The three gltA 97 Anaplasma sequences from deer differed by one nucleotide. The identity rates of the longest 98 sequence (729 bp) ranged from 87.9 to 98% with A. capra gltA sequences from Japan and China. 99 They were lower than 75% (61.4 to 74.6%) with *gltA* sequences from other *Anaplasma* species 100 (table 2). All these data confirmed the identity of the Anaplasma from French deer as belonging to 101 the A. capra species.

Partial sequences of the *16S* rRNA, *23S* rRNA, *groEL* and *gltA* from *A. capra* identified
from the swamp deer and one red deer were deposited in GenBank (accession numbers MH084717MH084724 with details in table 2).

105 Persistence of A. capra

The persistence of deer infection by *A. capra* was analyzed by sampling blood from one of the two infected red deer four months after the initial detection of this unexpected bacterial species. We detected *A. capra*, with 23S rRNA, 16S rRNA, groEL and gltA sequences 100% identical to the first identified *A. capra*, with the same distinct nucleotide in the gltA sequence as characterized 4 months earlier.

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112 **Discussion**

113 For about 40% of the positive conventional Anaplasmataceae spp. specific semi-nested 114 PCRs, sequences indicated amplification of *Ralstonia pickettii 23S* rRNA, highlighting a lack of 115 specificity of the semi-nested PCR used. As some of our negative controls were also positive, we 116 decided to sequence them and sequences corresponding also to Ralstonia picketii were found. As 117 this bacteria is a frequent contaminant of all kind of solutions, including ultrapure water [6], our 118 results most probably correspond to R. pickettii contamination of the solutions we used for 119 extraction or PCR. 120 In this survey, we detected two Anaplasma species infecting deer. A. phagocytophilum was 121 detected with a rather moderate prevalence (23.7%) in red deer only. Prevalence of A. 122 phagocytophilum infection in wild red deer in Europe is highly variable, from 1.5% in Austria, 123 10.9% in Portugal, 40-75% in Italy, 80.8% in Spain, to 97.9 to 100% in central Europe (respectively 124 in Slovakia and Hungary) [7-13]. Captive deer are probably less prone to tick bites compared to 125 wild ones, due to grazing areas management. This result indicates anyway the contact of red deer 126 with ticks and the transmission of A. phagocytophilum in the Reserve. Swamp deer were found 127 uninfected with A. phagocytophilum, a result which could be attributed to the low number of 128 animals analyzed in the case of a low infection rate (7). There are no data about tick-transmitted 129 pathogens for this endangered species, so the susceptibility of swamp deer to A. phagocytophilum is 130 unkown. A recently described Anaplasma species, A. capra was detected and identified in both deer 131 species, with a much lower infection rate (4.5%). A. capra has already been detected in various 132 ruminant hosts (sheep, goats, cattle, sika deer, Japanese serows) but its localization was up to now 133 geographically restricted to China and Japan [14-17]. Human infection by this newly-described 134 species has been reported in northeast China, leading to patients hospitalisation [2]. The detection 135 and characterization of A. capra based on several molecular markers in our study represents the first 136 evidence of this potentially new zoonotic species in Europe (France) in two new hosts, red deer and

137 swamp deer. Species assignation to *A. capra* was based on *16S* rRNA homologies higher than 99%
138 [18].

The 23S rRNA sequence from A. capra described in our study blasted with an unknown Anaplasma species proposed as "Candidatus Anaplasma mediterraneum" from sheep in Corsica (France)[5].
Whether "Candidatus Anaplasma mediterraneum" corresponds in fact to A. capra could not be determined, as the only other marker used in this study is *rpoB*, whereas we as most authors used a combination of *16S* rRNA, *groEL*, *gltA* and *msp4* sequences to identify and characterize Anaplasma (2, 15-17, 19-21).

145

146 We have detected A. capra in three different deer since 2015. The first infected and detected 147 red deer was a male originating from France (Theix) while the two others (red deer and swamp 148 deer) detected in 2017 and 2018 were both born inside the Park. Acquisition of a locally transmitted 149 A. capra is therefore probable for these two deer, even if A. capra may have been originally 150 introduced into the Park from an external source. The epidemiological cycle of A. capra seems 151 therefore to be completed locally. The low prevalence of infected deer in the Park might be due to 152 the introduction being recent. Ticks are the main vectors for Anaplasma species even if other 153 transmission routes have been described for some species (blood-sucking flies and transplacental 154 transmissions) [1]. Although A. capra has been detected in several tick species, *Ixodes persulcatus* 155 [2], Rhipicephalus microplus [19], Haemaphysalis longicornis [15,20] and Haemaphysalis 156 *qinghaiensis* [21], vectorial competence has not been proven yet. As most of these tick species are 157 not present in France, another tick species may be responsible for *A. capra* transmission in France. 158 The "Réserve de la Haute Touche" is located in a forested preserved area suitable for ticks and ticks 159 are commonly found feeding on the animals as well as questing on the vegetation (not shown). 160 Vector identification and vectorial competence remain to be elucidated. 161 In this study, we demonstrated the presence in France of the new species A. capra on two

162 new hosts. New studies are required, to examine its zoonotic ability, as non-zoonotic genetic

163	variants may exist as described in the case of A. phagocytophilum [1,3]. Diagnostic methods,
164	especially molecular ones, should take into account the potential infection of animals and humans
165	with this species, as molecular tools are often designed to specifically detect A. phagocytophilum.
166	To improve our knowledge on the epidemiological cycle of this bacterium in France, the vector tick
167	species should be identified, in order to evaluate the risks of transmission to humans. Deer could
168	therefore be considered as a potential reservoir for A. capra.
169	
170	Data Availability Statement
171	Gene sequences are available in GenBank under accession numbers MH084717-MH084724.
172	Competing interest
173	All authors report no conflicts of interest relevant to this article. There was no external funding for
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250 Table 1

251 Nucleotide sequence of primers used in the study

Target gene	Primer name	Sequence (5'-3')	Amplico	Reference
			n length	
23S rRNA	Ana23S-212F	ATAAGCTGCGGGGGAATTGTC	696 bp	[4]
	Ana23S-908R	TGGAGGACCGAACCTGTTAC		[4]
	Ana23S-212F	ATAAGCTGCGGGGGAATTGTC	541 bp	[4]
	Ana23S-753R	GTGACAGCGTACCTTTTGCA		[4]
16S rRNA	Ana16Sup1	CGGGTGAGTAATGCATAGGA	1089 bp	This study
	Ana16Sdo3	TAGCACGTGTGTAGCCCAC		This study
	Ana16sIntup1	AACTCCGTGCCAGCAGCCGCG	581 bp	This study
	Ana16Sdo1	CCCAACATCTCACGACAC		This study
gltA	Outer-F	GCGATTTTAGAGTGYGGAGATTG	1077 bp	[2]
	Outer-R	TACAATACCGGAGTAAAAGTCAA		[2]
	Inner-F	GGGTTCCTGTC <u>C</u> ACTGCTGCGTG	793 bp	[2]*
	Inner-R	TTGGATCGTA <u>A</u> TTCTTGTAGACC		[2]*
groEL	Ac-groEL-F1	GCGAGGCGTTAGACAAGTCCATT	1264 bp	[2]
	Ac-groEL-R3	TCCAGAGATGCGAGCGTGTATAG		[2]
	Ac-groEL-F2	TGCACTGCTGGTCCAAAGGGGGCT	1087 bp	This study
	Ac-groEL-R2	CAACTTCGCTAGAGCCGCCAACC		This study

• Modified from [2]*, in accordance to GenBank accession number KM206274 sequence

255 Table 2

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57	Sequence	lengths and	l identity rates	with Anapl	lasma spp.	sequence	36

Gene	Sequence lengths	GenBank accession numbers	Reference organisms and sequences	Identity rate
23S rRNA	CEL15 - 367 bp CEL17 - 506 bp DVC 17 - 515 bp	MH084724 MH084723	Cand. A. mediterraneum KY498330 A. ovis KM021411 A. centrale NR-076686 A. marginale KY498332 A. platys KM021412 A. phagocytophilum KM021418	99.6 % 95.0% 94.6% 93.8% 91.6% 90.8%
<i>16S</i> rRNA	CEL17 - 531 bp DVC 17 - 518 bp	MH084721 MH084722	A. capra KM206273 - MF066917 A. marginale AF414874 A. centrale AF318944 A. ovis AJ633049 A. phagocytophilum NR-044762 A. platys AY077619	99.6% - 99.8% 98.7% 98.5% 98.3% 96.4% 96.2%
groEL	CEL15 - 559 bp CEL17 - 1008 bp DVC 17 - 1087 bp	MH084718 MH084717	A. capra KM206275 - AB454078 A. marginale AF414864 A. centrale EF520691 A. ovis AF441131 A. platys AY044161 A. phagocytophilum JF494833	91.4% - 97.7% 83.2% 83.2% 83.2% 77.5% 76.2%
gltA	CEL15 - 729 bp CEL17 - 707 bp DVC 17 - 725 bp	MH084720 MH084719	A. capra KM206274 - MG940872 A. marginale AF304139 A. ovis PKOE01000003 A. centrale CP001759 A. phagocytophilum AY464132 A. platys EU516387	87.9% - 98% 74.6% 74.2% 73.3% 65.4% 61.4%

259 CEL : Cervus elaphus

260 DVC : Rucervus duvaucelii