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1 Diagnosing plague in 17th century camelids from Romania: unmasking role of
2 camelids in plague dissemination along historical Silk Routes.

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4 Hamadou Oumarou Hama^{1,2}, Mahmoud Boualam¹, Jean Armengaud³, Michel
5 Drancourt^{1,2}, Gérard Aboudharam^{1,2,4}, Adrian Bălăşescu⁵, and Valentin Radu⁶

6
7
8 1. IHU Méditerranée Infection, Marseille, France.

9 2. Aix-Marseille Université, MEPHI, IHU Méditerranée Infection, Marseille,
10 France.

11 3. Université Paris-Saclay, CEA, INRAE, Département Médicaments et
12 Technologies pour la Santé (DMTS), SPI, 30200 Bagnols sur Cèze, France.

13 4. Ecole de Médecine Dentaire, Aix-Marseille Université, Marseille, France.

14 5. “Vasile Pârvan” Institute of Archaeology, Romanian Academy, Bucharest,
15 Romania.

16 6. University of Bucharest, ArchaeoSciences Division, ICUB, Bucharest,
17 Romania.

18
19 Corresponding author: Hamadou OUMAROU HAMA, MEPHI, IHU Méditerranée
20 Infection, 19-21 Boulevard Jean Moulin, 13385 Marseille, cedex 05, France.
21 hamadouh2o@gmail.com.

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27 **Abstract**

28 Six camels (R01-R06) exhumed from a 17th century Silk Route site in Agighiol,
29 Romania, along with negative controls, were blindly investigated via dental pulp
30 paleometagenomics and paleoproteomics for traces of *Yersinia pseudotuberculosis*
31 complex including the plague agent *Yersinia pestis*. Sample R04 yielded a 32 bp
32 read specific for the *Y. pseudotuberculosis* complex valine-tRNA ligase gene;
33 sample R05 yielded a 30 bp and a 33 bp reads specific for the *Y. pseudotuberculosis*
34 complex. Further, samples R04 and R05 yielded a 16S rRNA guanine transferase
35 specific for *Y. pestis* and one other *Y. pseudotuberculosis* complex peptide. Taken
36 together, these validated data diagnosed plague in these ancient camels which likely
37 participated in plague dissemination along ancient Silk Routes during the large
38 Medieval and Modern Times pandemic in Europe.

39

40 Plague is a deadly zoonosis caused by *Yersinia pestis* (*Y. pestis*), which is endemic
41 to 33 tropical countries in Africa, Asia and the Americas, with the majority in
42 Madagascar, the Democratic Republic of the Congo and Peru [1]. Europe is currently
43 a plague-free continent after having been severely affected for millennia. Notably,
44 the Black Death epidemic, which started in 1346 before vanishing by the mid-18th
45 century, claimed an estimated 25 million lives (approximately one-third of the
46 medieval population in Europe) and dramatically impacted the political and
47 sociological landscape of the continent [2]. The Black Death reached Europe through
48 Mediterranean Sea ports due to the trading of silks, furs, and other goods that were
49 exported from the Far East and China along the terrestrial commercial routes known
50 as the Silk Routes. However, the contributions of caravan members, particularly
51 camels, to plague propagation along the long Silk Routes remains poorly understood
52 [3].

53

54 **Results**

55 The site of Agighiol, Romania (Doubroudja region) is exceptional because a large
56 number of camelid remains (n = 155) were identified: To the best of our knowledge,
57 only two other examples of camel remains had been previously identified from
58 archaeological settings in the territory of Romania [4]. The large number of camels
59 remains were accidentally discovered in 2008 during construction work on a private
60 property, in the center of this village, in an area with no previously recorded
61 archaeological findings [5]. The village of Agighiol (Tulcea county) was mentioned by
62 Evlia Celebi (Adgi-gol) in 1657, being populated by Turks and Tatars and presence
63 of camels in this period could be linked to movements of some populations (Tatars)
64 from the North-East of the Black Sea or to the caravans linking the Orient with

65 Europe [6]. This site used to be a caravanserai for caravans coming from China and
66 Far East along one Silk Route acknowledged as having been also one plague route
67 [4]. The 155 camelid remains issued from six adults (at least three *Camelus*
68 *bactrianus* individuals, one of *Camelus dromedarius*, one hybrid individual and one
69 unidentifiable individual – *Camelus* sp.) from that site gave the opportunity to
70 investigate traces of zoonotic plague in six of these animals, using two neutral,
71 untargeted experimental approaches applied to the dental pulp. As a first step,
72 metagenomics yielded $9,151,722 \pm 15,788,486$ reads per sample (with 1,038,702 in
73 the blank) including three reads of interest absent from blank (Fig. 1): In sample R04,
74 one 32 bp read (5'- TACTGCATCATGATCCCGCCGCCGAACGTGAC-3') detected
75 in 1 out of 256 consensus reads was subjected to a BLAST search and showed
76 100% sequence similarity to the *Y. pseudotuberculosis* and *Y. pestis* valine-tRNA
77 ligase (valS) gene. In sample R05, two 30 bp and 33 bp reads (respectively, 5'-
78 GCGATGGTTTATTCGGTGATTGTGGTACCG-3' and 5'-
79 GTGCCAACCACGTCGGTGACTGGGGTACTCAGT-3') detected in 2 out of 271
80 consensus reads showed 100% sequence similarity to the *Y. pseudotuberculosis*
81 and *Y. pestis* NCS2 family permease and arginine-tRNA ligase (argS) genes (Fig. 1).
82 These results prompted us to employ orthogonal proteotyping using high-resolution
83 tandem mass spectrometry and shotgun paleoproteomics (Supplementary data 1),
84 which confirmed the camelid origin of the teeth through the identification of 240
85 distinct peptides assigned to *Camelus* species. Strikingly, the QLSDNLMLLK peptide
86 indicating the presence of a 16S rRNA guanine (966)-N(2)-methyltransferase from *Y.*
87 *pestis* was detected in both its oxidized and nonoxidized forms in samples R02, R03,
88 R04 and R05, whereas no trace of this peptide was detected in the blank negative
89 control. The corresponding doubly charged ions at m/z 596.3181 and 588.3208 were

90 thus confidently assigned with peptide mass errors of 0.0 and 0.3 ppm, respectively.
91 These signals were particularly abundant in R05, with 10 assigned spectra
92 (Supplementary Table S1). Another peptide, YQEVAQK which is universal in *Y.*
93 *pseudotuberculosis* complex was also identified.

94

95 **Discussion**

96 Present data derived from two independent and orthogonal methodologies, both of
97 which were conducted with negative controls, indicate that at least two individual
98 camels (R04 and R05) were infected with *Y. pestis*, diagnosing plague in both
99 camelids. Moreover, the involvement of dental pulp further indicates a deadly
100 septicemic plague [7].

101 These two camels have been retrieved from a 17th century caravanserai site
102 documented as part of Silk Routes connecting China and Far East countries to
103 Europe for millennia; passing through countries where *Y. pestis* has been detected in
104 ancient human teeth from Asia and Europe dating from 2,800 to 5,000 years ago [8].
105 Indeed, camelids have been historically documented along the Silk Routes, being
106 stocking animals which were eventually skinned and eaten after their death,
107 including plagued animals: Historical documents have indicated that camels were
108 butchered in Europe until the 19th century [9].

109 At present, plague is transmitted between camels via direct contact and lice
110 [3], making *Y. pestis*-infected camels deadly for populations in close contact when
111 these animals are skinned or when their meat or livers are raw or poorly cooked and
112 eaten, particularly in Russia, Mongolia, Saudi Arabia, Jordan, Libya and Algeria [10-
113 12]. This mode of contamination can even lead to enteritis, a rare presentation of this
114 deadly infection [13]. Therefore, we suggest that plagued camels may have been a

115 neglected source of plague among medieval merchants along the Silk Routes and in
116 European populations. The aphorism that plague follows man should become plague
117 also follows camels.

118

119 **Materials and Methods**

120 Here, six teeth (reported as R01-R06) collected from 17th century (Agi 1–2 sigma,
121 1643 AD:1669 AD, 0.63 probability; and Agi 2–2 sigma, 1622 AD:1666 AD, 0.57
122 probability) camelids retrieved in Agighiol, Tulcea County, Romania (GPS
123 coordinates 45°02'10.1"N 28°52'33.6"E) (4,5) were investigated via direct total
124 metagenomics (European Nucleotide Archive project PRJEB81205) and proteomics
125 (datasets PXD056179 and 10.6019/PXD056179) (Fig. 1). Ancient DNA (aDNA)
126 sequenced on a NovaSeq 6000 instrument (Illumina Inc., San Diego, CA, USA)
127 following the Illumina COVIDSeq protocol, as described elsewhere [14] in the
128 presence of a blank negative control. Reads were mapped against two reference
129 genomes (CP002956.1: *Y. pestis* A1122 and CP009712.1: *Y. pseudotuberculosis* IP
130 32953) using CLC Genomics Workbench software (version 7.5), with a 0.95
131 similarity fraction, 0.8 length fraction, deletion cost of 3, insertion cost of 3, and
132 mismatch cost of 2. was subjected to a BLAST search.

133

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169 **Figures**

170 **Figure 1. Summary of the metagenomic and paleoproteomic results.**

171 Identification and alignment of three aDNA reads and two *Yersinia*
172 *pseudotuberculosis* complex peptides retrieved from camel dental pulp using a
173 database comprising the *Camelus dromedarius* annotated genome
174 (GCF_036321535.1), and all available *Y. pseudotuberculosis* and *Y. pestis*
175 annotated NCBI genomes (downloaded 2024-05-27), comprising 243,778 protein
176 sequences and totaling 95,820,792 amino acid residues. A: Three reads of 30–33 bp
177 from *Y. pseudotuberculosis* / *Y. pestis* obtained by metagenomic analysis of the
178 dental pulp of camels R04 and R05 (the starting and ending positions of *VaIS*, *NCS2*
179 and *argS* genes relative to the two *Yersinia* reference genomes (YRG): CP002956.1:
180 *Y. pestis* A1122 in pink and CP009712.1: *Y. pseudotuberculosis* IP 32953 in
181 orange). B: Two peptides from *Y. pseudotuberculosis complex* / *Y. pestis* obtained
182 by paleoproteomic analysis of the dental pulp of camels R04 and R05 (the starting
183 and ending positions relative to the *YdbL* and *rsmD* proteins relative to the two
184 *Yersinia* reference proteins (YRP): WP_002211001.1: *Y. pseudotuberculosis*
185 *complex* in blue and WP_012229076.1: *Y. pestis* in green).

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198 **SUPPLEMENTARY DATA**

199 **Supplementary Materials and Methods**

200 **Paleoproteomics**

201 Proteins (30 μ L of extract) were reduced with 5 μ L of 35 mM dithiothreitol and
202 alkylated with 5 μ L of a 105 mM iodoacetamide/100 mM NH_4HCO_3 solution for 10
203 min at room temperature in the dark. Then, the modified proteins were captured with
204 200 μ g of SP3 Sera-Mag SpeedBeads (Sigma–Aldrich) prepared as a 50 mg/mL
205 solution with a 1:1 mixture of hydrophilic (Cytiva 45152105050250) and hydrophobic
206 (Cytiva 65152105050250) carboxylate magnetic particles and then treated with 250
207 μ L of acetonitrile for 2 min at room temperature. The bead–protein complexes were
208 collected with a magnet and washed twice with 200 μ L of 70% ethanol and once with
209 180 μ L of CH_3CN . The proteins were then digested with 30 μ L of 0.1 μ g/ μ L Trypsin
210 Gold (Promega) in 50 mM NH_4HCO_3 supplemented with 0.01% ProteaseMAX
211 surfactant (Promega) for 30 min at 50°C. The resulting peptides were desalted using
212 an Attract SPE Disk SpinC18 device (Affiniseq) as recommended by the supplier.
213 The peptides were acidified with 0.5% trifluoroacetic acid and then injected on a
214 reversed-phase PepMap 100 C18 precolumn (5 μ m, 100 Å, 300 mm i.d. \times 5 mm,
215 Thermo Scientific) and separated with an EasySpray PepMap 100 Neo C18 column
216 (2 μ m, 100 Å, 75 mm i.d., \times 50 cm, Thermo Scientific) in a Vanquish Neo UHPLC
217 system (Thermo Scientific) at a flow rate of 25 nL/min using. The mobile phases
218 were A (0.1% HCOOH in 100% H_2O) and B (0.1% HCOOH in 100% CH_3CN), and
219 samples were eluted according to the following gradient elution program: 5%–25% B
220 over 30 min, followed by 25%–40% B over 5 min. The outlet of the column was
221 coupled to an Orbitrap Exploris 480 high-resolution mass spectrometer (Thermo
222 Scientific) operated in data-dependent acquisition mode with full-scan mass spectra

223 acquired from *m/z* 350 to 1500, as previously described (S1). Only peptides with two
224 or three positive charges were fragmented (Top10 method), with dynamic exclusion
225 set to 5 sec and an intensity threshold of 2,000. Peptide sequences were identified
226 from the MS/MS spectra using the Mascot Daemon 2.6.1 search engine (Matrix
227 Science) and a database comprising the *C. dromedarius* annotated genome
228 (GCF_036321535.1) and all available *Y. pseudotuberculosis* & *Y. pestis* annotated
229 NCBI genomes (downloaded 2024-05-27), comprising 243,778 protein sequences
230 and totaling 95,820,792 amino acid residues. The recommended parameters for
231 modifications, carbamidomethylation of cysteines, deamidation of asparagine and
232 glutamine, and oxidation of proline and methionine, were applied (S2). Peptides and
233 proteins were identified with a false discovery rate of 1%.

234

235 **NovaSeq 6000 sequencing data**

236 Metagenomic sequence data are available from the European Nucleotide Archive
237 (ENA: <https://www.ebi.ac.uk/ena/browser/home>) under accession project
238 PRJEB81205/ ERP165049. Data will be made public from 25 March 2025.

239

240 **Mass spectrometry data**

241 The mass spectrometry proteomics data are available from the ProteomeXchange
242 Consortium via the PRIDE partner repository under dataset identifiers PXD056179
243 and 10.6019/PXD056179. [The reviewers may access this currently private dataset
244 using reviewer_pxd056179@ebi.ac.uk as the username and KtLAMfcqNFy8 as the
245 password].

246

247 **Supplementary references**

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254
255 Supplementary Table S1.