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An ancient coronavirus from individuals in France, circa 16th century

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ABSTRACT

Objectives: At the time when the COVID-19 pandemic was responsible for more than six million deaths worldwide, the antiquity of coronaviruses remains undefined. We investigated individuals buried during the 16th century in France for the direct and paleoserological diagnosis of the coronavirus.

Methods: The 2011–2012 excavation of Abbey Saint-Pierre in Baume-Les-Messieurs, France uncovered 12 skeletons of individuals from the 13th to the 18th century. The total proteins extracted from dental pulps were subjected to microbial paleoserology, targeting SARS-CoV-2, human-associated coronavirus (HCoV)-229E, and OC43 antigens and for coronavirus peptide research using metaproteomics, in parallel to negative controls.

Results: Three peptide sequences totaling 36 amino acids indicative of a coronavirus were retrieved from the dental pulp remains collected from two individuals buried circa 16th century, in whom paleoserology confirmed a specific immunological response against modern-day SARS-CoV-2 and HCoV-229E.

Conclusion: We provide serological and proteomic evidence for a betacoronavirus with no modern correspondent, infecting populations in the 16th century, extending the antiquity of coronaviruses by more than three centuries. Historical, archaeozoological, and paleoproteomic data suggested close contacts between these two individuals and domestic swine, cattle, and poultry, suggesting an ancient zoonotic coronavirus. Coronaviruses have been undesirable companions of populations long before the ongoing coronavirus disease 2019 outbreak emerged.

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INTRODUCTION

The ongoing COVID-19 pandemic, responsible for more than six million deaths worldwide from 2019 to 2022 [1,2], is the latest representative of a series of coronavirus epidemics that have affected different populations for decades, after the severe acute respiratory syndrome coronavirus (SARS-CoV) epidemics claimed 8096 probable cases and 774 associated deaths (9.6%) between November 2002 and July 2003 [3], and Middle East respiratory syndrome coronavirus claimed 2519 confirmed cases and 866 associated deaths (34.3%) between 2012 and January 2020 [4]. In addition, human coronaviruses (HCoVs) have been acknowledged as the pathogens responsible for the common cold and upper respiratory tract disease frequently occurring as outbreaks for more than

half a century [5]. Coronaviruses named the B814, 229E, and OC series were detected in the mid-1960s from upper respiratory tract samples using tracheal organ cell cultures [6–8], but contemporary electron microscopy observations of human samples revealed the unique crown-like morphology of coronaviruses [9]. More recently, the discovery of HCoV-NL63 [10,11] and HCoV-HKU1 in 2004–2005 [12] enriched the spectrum of known HCoVs. Further studies characterized these various HCoV among two main genomic groups, which include alphacoronavirus in humans, which include HCoV-229E and HCoV-NL63, and betacoronavirus in humans, which include the Middle East respiratory syndrome coronavirus, SARS-CoV, HCoV-OC43, and HCoV-HKU1 [13,14]. In addition, porcine respiratory coronavirus, feline coronavirus, canine coronavirus, bovine coronavirus, and porcine delta coronavirus characterized in animals have not been associated with humans [13,14]. These different groups of coronaviruses nevertheless share common characteristics, including a large RNA genome of average 30 kilobases coding for antigenically cross-reactive proteins, among which the

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Figure 1. Localization, burial, dating and age information about individuals buried in the choir of the Saint-Pierre abbey, Baume-Les-Messieurs, France (Panel a), and one individual buried in Viotte Nord, Besançon, France (Panel b). Figures a and b are adapted and reproduced from the original archaeological report produced by Bully and Bassi [18,29] with their kind authorization.

Abbreviations: A, age; C¹⁴, carbon-14; D, date; Ind., individual; S, sex.

respective spike proteins and nucleocapsid proteins have been previously found to be immunodominant [14–16].

However, little is known regarding the antiquity of HCoV beyond one preliminary report by our research team on the detection of anti-HCoV antibodies in five World War I soldiers who died in 1914 in Spincourt, France, aged between 20 and 50 years [17]. The current study further extends the antiquity of HCoVs by more than three centuries after we detected pieces of peptide sequences, as well as corresponding HCoV-specific antibodies in two buried individuals in France.

METHODS

Archaeological materials

In 2011–2012, the excavation of the former Abbey Saint-Pierre choir in Baume-Les-Messieurs (a small village in Jura department, eastern France: 46° 42′ 31″ North, 5° 38′ 57″ East) yielded 12 skeletons from the 13th to 18th century, as estimated by architectural, furniture, and historical data and C¹⁴ radiocarbon dating of one bone sample collected from grave backfill surrounding the 12 individuals (Figure 1) [18].

These 12 individuals were all identified as men, as determined using the Bruzek method [19], and determined to be aged 30–60 years at the time of death based on the Schmitt method [20,21]. In the current study, dealing with dental pulp as an ancient sample on which to base paleomicrobiological investigations [22], mandibles and teeth were recovered from each individual, except for uncollected individual three and toothless individual 10. The teeth and mandibles collected on site in

2012 were stored at ambient temperature in Besançon Archaeological Center, France until 2018 and further sent to the IHU Méditerranée Infection, Marseille, France, in accordance with French regulations for archaeological studies. Mandibular and tooth reception, storage, and manipulation occurred between spring 2018 and December 2020 in successive laboratory facilities, where SARS-CoV-2 had never been manipulated (Supplementary Figure 1).

Finally, dental pulps recovered following the previously described methods [23] were pooled per individual and stored at room temperature in closed sterile microtubes (Biosigma, Cona, Italy) for further analyses, as described subsequently. In addition, radiocarbon dating was performed on the tooth remains collected from individuals 5 and 12 (Beta Analytic, Madrid, Spain). In addition, a large amount of faunal material (9955 g in total) was found in 34 different stratigraphic units from the soil or culinary discards [18].

Prevention of contamination

Contamination being of particular concern in paleomicrobiology [24], the authors enforced all available process and techniques to prevent and control the absence of contamination of the ancient material herein studied (Supplementary Figure 1). The storage of archaeological samples was done in a dedicated closed cabinet, and the recovery of dental pulp and protein extraction were done under a hood exclusively dedicated to the handling of archaeological samples, in which no modern samples were ever manipulated. Each tool used for pulp recovery was autoclaved beforehand and each reagent used as described in our protocol was new and care-

fully decontaminated as previously reported [25]. We have carefully included for each experiment, paleoserology and paleoproteomics, in addition to our negative controls, at least two blanks (extraction controls) per experiment that were handled under the same conditions and at the same time as our samples to prevent any contamination by the operators. None of the operators was known to be infected by any coronavirus at the time of the experiments.

Paleoserology and paleoproteomics

For each of the pooled dental pulps (three dental pulps pooled per individual for 10 individuals under investigation), protein extraction was performed as previously described without any modification to yield a so-called paleoserum solution [26]. For paleoserology, 20 µl of the paleoserum solution was incorporated into a mini-line blot assay that we have previously described for the pathogen-specific detection of antibodies in paleosera, as shown subsequently [26]. Noninoculated MRC-5, HCT8, and Vero cell lines, as well as cell lines inoculated with alphacoronavirus HCoV-229E, betacoronavirus OC43, and betacoronavirus SARS-CoV-2 were verified by reverse transcription-polymerase chain reaction (bioFire FilmArray, bioMérieux, UT, USA) and confirmed by Western immunoblotting (Jess Blot, ProteinSimple, San Jose, CA, USA) before antigen production. In addition, pan-*Mycoplasma* polymerase chain reaction was performed before and after the antigen production to confirm the lack of mycoplasma infection of cells. Then, thus validated SARS-CoV-2, HCoV-229E, and OC43 antigens; noninoculated HCT8, MRC5, and Vero cells; *Staphylococcus aureus* antigen used as a positive control; and skim milk as a negative control were incorporated onto a nitrocellulose membrane (Bio-Rad, Marnes-la-Coquette, France). Nitrocellulose strips were incubated with 20 µl of paleoserum diluted in saturation buffer (PBST-M 0.5%, 1:1000) at room temperature for 2 hours under agitation before incubation with peroxidase-conjugated AffiniPure Goat Anti-Human immunoglobulin (Ig)A + IgG + IgM (H + L) (Jackson ImmunoResearch, West Grove, PA, USA). The results were quantified using a FUSION FX chemiluminescent imaging system and ImageQuant TL software, as previously reported [26]. Furthermore, 20 µl of the paleoserum solution collected from individuals BA-I5 and BA-I12 who tested seropositive for coronavirus antibodies were processed for ancient peptide detection (ProGénoMIX platform, CEA, Marcoule, France) by electrophoresis to split the total proteome into five equal fractions differing by their molecular weight, treated and proteolyzed as previously described [27]. Then, 10 µl (one-fifth) of each tryptic fraction was analyzed with an Exploris 480 tandem mass spectrometer (ThermoFisher Scientific, Illkirch-Graffenstaden, France), incorporating an ultra-high-field Orbitrap analyzer and connected to an UltiMate 3000 LC system (ThermoFisher Scientific). To gain sensitivity, an iterative analysis of each of the fractions was performed with its specific list of exclusion. An original metaproteomics cascade search strategy was used to assign MS/MS spectra to peptide sequences with the Mascot Daemon 2.6.1 search engine (Matrix Science). Briefly, an National Center for Biotechnology Information (NCBI)nr-derived database containing 45,925 representative taxa as previously described [28], a database restricted to the identified genera, and a database of identified proteins and viruses were successively queried (Supplementary Figure 2). Peptides and proteins were identified with a false discovery rate of 1%, including the deamidation of asparagine and glutamine and oxidation of proline and methionine as variable modifications. In parallel, 20 µl of the paleoserum solution collected from individuals BA-I7, BA-I9, and BVN-293, from whom the samples were collected from another archaeological site in Viotte Nord, Besançon, France [29,30] dating from the 2nd to 3rd century and tested seronegative for coronavirus were processed.

RESULTS

Archaeological materials

Four of 10 tombs examined in the current study dated from the mid-15th to 18th century, located in the eastern part of the choir hosted men aged over 60 years and lying on their backs oriented west-east, with heads to the west; all were buried in coffins, as illustrated by traces of wood and nails and decomposition into empty space or spaces semipacked with lime. Furthermore, nine tombs dated from the 13th to 16th century located all over the choir hosted males aged over 30 years, including individual four who was aged over 40 years, all lying on their back oriented west-east, with the head to the west, except for individuals five and 12 oriented east-west with the head to the east; all were buried in a coffin, except for individuals three and four (Figure 1). Further skeleton examinations revealed mainly age-related pathologies, including osteoarthritis, ectopic ossification, and bone porosity and a diffuse idiopathic skeletal hyperostosis in individual seven. In addition, the dentition analysis indicated antemortem tooth loss affecting almost all the subjects in agreement with mature age and suggested a rather poor sanitary state, regardless of the historical period. Regarding faunal material, there was an overrepresentation of swine (4913 g, i.e., 49.3% of total faunal material) over beef (1815 g, i.e., 18.2%), deer (757 g, i.e., 7.6%), poultry/birds (686.5 g, i.e., 6.8%), and fish [18]. Additional radiocarbon dating of individuals BA-I5 and BA-I12 indicated 1474–1638 calAD and 1540–1634 calAD, respectively (Supplementary Figure 3).

Absence of contamination

All the negative controls and blanks introduced in the experiments, as explicated in the Materials & Methods section, remained negative, i.e., absence of antibody/antigen reaction and absence of peptide sequence having at least 90% identity with a coronavirus. The negative results were interpreted as the absence of any contamination of the experimental archaeological materials, thus validating the positive observations here reported.

Paleoserology

After the coronavirus antigens were certified as previously mentioned, paleoserology was interpretable for 1- paleosera investigated here because the skim milk negative control remained negative, whereas the *S. aureus* positive control reacted with all the paleosera. Under these conditions, the paleosera collected from eight individuals remained free of any detectable reactivity against any one of the three coronavirus antigens introduced in the mini-line blot assay, whereas the paleosera collected from individual BA-I5 and from individual BA-I12 reacted against SARS-CoV-2, HCoV-229E, and OC43 antigens, and the paleoserum collected from individual BA-I5 reacted against HTC8 cells (in which coronavirus OC43 antigen had been produced). The quantification of the paleoserological assay yielded arbitrary units (AUs) ranging from 0 AU to 2,125,874.79 AU; indicating that individuals BA-I12 and BA-I5 showed reactivity marked by an area under the curve > 130,000 AU against the three coronavirus antigens. In detail, the paleoserum retrieved from individual BA-I12 yielded 520,888.32 AU against SARS-CoV-2, 413,582.97 AU against HCoV-229E, and 170,029.19 AU against HCoV-OC43, whereas the paleoserum retrieved from individual BA-I5 yielded 721,907.39 AU against SARS-CoV-2, 311,342.81 AU against HCoV-229E, and 130,863.28 AU against HCoV-OC43, as well as 291,125.25 AU against HTC8 cells, which supported the growth of the HCoV-OC43 antigen, limiting the interpretation of paleoserum reactivity against the HCoV-OC43 antigen (Figure 2).

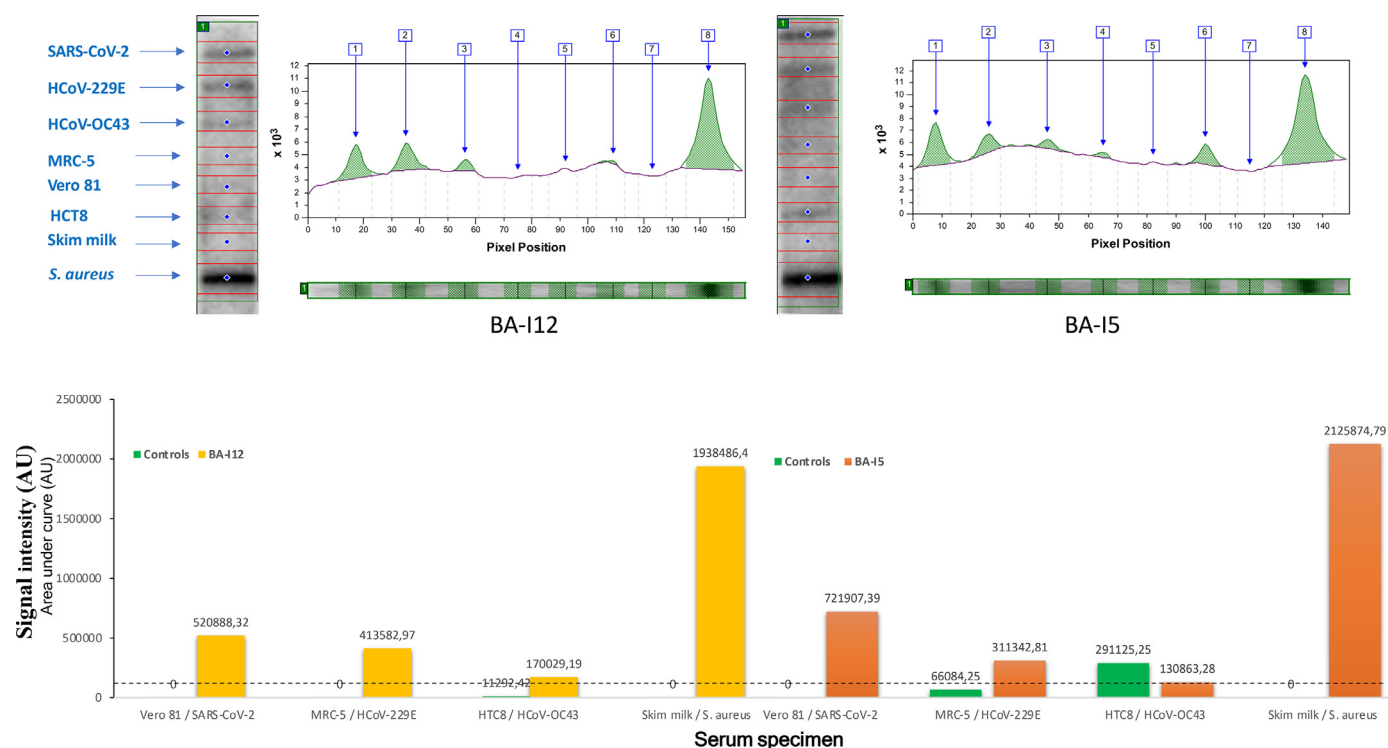


Figure 2. Paleoserological investigations of the two individuals BA-I5 and BA-I12 buried circa 16th century, using a mini-line blot assay [26] incorporating three coronavirus antigens for SARS-CoV-2, HCoV-229E and HCoV-OC43; MRC5, Vero 81, and HTC8 cells and skim milk as negative controls; and *Staphylococcus aureus* as a positive control. Upper panel, densitogram of the serological reactions. Lower panel, quantification of reactions, in AU (The cut-off of 130,000 AU was represented by a dotted line). Abbreviations: AU, arbitrary units; HCoV, human coronavirus.

Paleoproteomics

Mass spectrometry analyses produced 93,984 MS/MS spectra sorted into 976 peptides in the BA-I7 sample, 95,882 MS/MS spectra sorted into 1023 peptides in the BA-I9 sample, and 41,958 MS/MS spectra sorted into 1051 peptides in the BVN-293 sample, all three of which were negative controls. Furthermore, 153,053 MS/MS spectra were sorted into 3634 peptides in the BA-I5 sample, and 154,815 MS/MS spectra were sorted into 3563 peptides in the BA-I12 sample. The higher numbers of peptide sequences observed for the two later samples is due to differences of the dynamic range of the proteomes and the resulting diversity of the peptidomes. The ancientness of these peptides was supported by the extent of proline hydroxylation representing 1057 (14.5%) and 990 (13.7%) peptide-to-spectrum matches in individuals BA-I5 and BA-I12, respectively, and pointing at a large diversity of proteins. The ratio of oxidized proline residues is in the same range with 15.2% for BA-I5 and 13.6% for BA-I12. The ratio of oxidized proline residues was relatively stable over the five samples (13.6–23.4%), whereas the ratios of deamidated asparagine and glutamine residues and oxidized methionines are more variable (5.1–34.6% and 41.6–84.4%, respectively) (Supplementary Table S1). Furthermore, the BA-I5 sample contained 490 taxon-specific peptides related to *Homo sapiens* and 81 taxon-specific peptides related to *Bos taurus*, including casein, lactoglobulin, and lactotransferrin proteins, whereas the BA-I12 sample contained 443 taxon-specific peptides related to *H. sapiens* and 82 peptides related to *B. taurus*. Subsequently, the paleoproteomics did not reveal any peptide related to coronaviruses in the three investigated seronegative individuals BA-I7, BA-I9, and BVN-293, whereas such peptides were detected in seropositive individuals BA-I5 and BA-I12. In total, three peptides recovered from individuals BA-I12 and BA-I5 yielded 100% coverage and 100% identity exclusively with coronavirus homologous sequences: individ-

ual BA-I12 yielded a 9-residue (IVLSEYKK) peptide that was observed in 9 MS/MS spectra in the extended dataset, corresponding exactly to the polyprotein replicase 1a of the alphacoronavirus porcine respiratory coronavirus (start_end positions: 912 to 938, NCBI accession number KY406735.1), swine enteric coronavirus (start_end positions: 904 to 930, NCBI accession number KR061459.1), and canine coronavirus (start_end positions: 911 to 937, NCBI accession number MW591993.2), and a 17-residue (RGPEQTQGNFGDQELIR) peptide observed in three MS/MS spectra, a peptide sequence shared within the nucleocapsid protein of the betacoronavirus SARS-CoV-1 (start_end position: 28,680 to 28,730, NCBI accession number KJ473816.1), SARS-CoV-2 (start_end position: 29,102 to 29,152, NCBI accession number NC_045512.2), and the bat *Rhinolophus affinis* coronavirus (start_end position: 29009 to 29059, NCBI accession number KF569996.1). For individual BA-I5, the analyses yielded a 9-residue peptide (IVLSEYKK) observed in 11 MS/MS spectra, which is also found within the polyprotein replicase 1a sequence described previously; a 17-residue (RGPEQTQGNFGDQELIR) peptide in four MS/MS spectra of nucleocapsid protein, as described previously; and a 10-residue (AYNVTQAFGR) peptide observed in two MS/MS spectra, most closely related to the nucleocapsid peptide of SARS-CoV-2 (start_end position: 29,072 to 29,101, NCBI accession number NC_045512.2), *R. affinis* coronavirus (start_end position: 28,979 to 29,008, NCBI accession number KF569996.1), and the bat coronavirus BM48-31/BGR/2008 (start_end position: 28454 to 28483, NCBI accession number NC_014470.1) (Figure 3).

DISCUSSION

A total of 3 years ago, Chinese physicians reported in the *New England Journal of Medicine* three patients who developed infection with a previously unknown betacoronavirus currently referred to

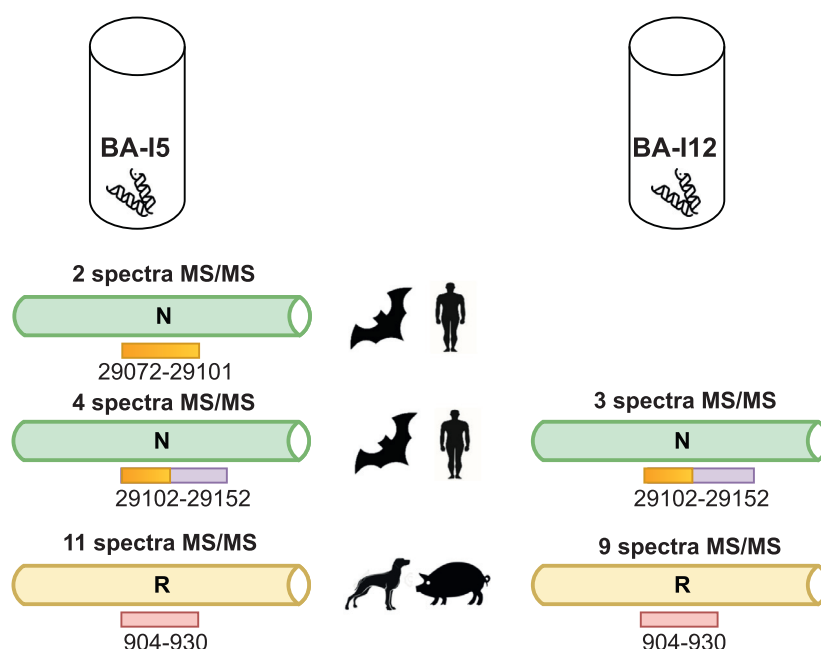


Figure 3. Summary of paleoproteomic investigation and identification and alignment of the three coronavirus peptides retrieved from individuals BA-I5 and BA-I12 using two representative coronavirus genomes (NCBI accession numbers KR061459.1 and NC_045512.2). N: Coronavirus nucleocapsid peptide (start_end positions relative to the Wuhan-Hu-1 genome sequence: 29072 to 29152); R: replicase polyprotein 1a peptide (start_end positions relative to the swine enteric coronavirus strain genome sequence: 904 to 930).

as SARS-CoV-2 [1]. Here, we report on two individuals buried circa 16th century in the Abbatale Saint-Pierre archaeological site in Baume-Les-Messieurs in northeastern France who were diagnosed with coronavirus infection after coronavirus-specific peptides and anticoronavirus antibodies were detected in the presence of negative controls and remained undetected in eight other individuals buried in the same site. The absence of pathological data precluded any conclusion regarding the potential illness and lethal role of this coronavirus in these two individuals, for whom limited biographical data are known, yet the paleoanthropological analysis indicates that they were male individuals who died after 30 years. Their probable monastic function is suggested by their inhumation into a coffin in the choir of the abbey.

These unprecedented observations were made possible after investigating the ancient dental pulp specimens, which contain conserved blood elements of the host at the time of death [30], allowing the detection of both immunoglobulins and pathogen peptides. The recovered peptides indicated a coronavirus that was different from any modern coronavirus, including SARS-CoV-2, suggesting an out-of-age coronavirus with no known modern representative.

Archaeological and paleoproteomic data obtained after the analysis of positive individuals showed that these individuals were in close contact with wild mammals, such as deers (*Cervus elaphus*), and domesticated mammals, such as swine (*Sus scrofa*), cattle (*B. taurus*), dogs, and poultry; all these animal species have been acknowledged as reservoirs of coronaviruses that can also infect human populations [31–35].

Paleoproteotyping has proven to be a powerful approach to identify the taxonomic origin of ancient organic materials [36,37]. Here, we have shown that viruses can be traced with tandem mass spectrometry. The main potential weakness of this analytical approach is the completeness of the generalist database used, which should improve over time with continued sequencing of representatives from all branches of the tree of life.

This report of what could be considered as the oldest known HCoV infection will hopefully pave the way for additional reports combining the methods reported here to trace the antiquity and

dynamics of coronavirus infection in past populations, giving a deeper scientific perspective to the current COVID-19 pandemic.

Declaration of competing interest

The authors have no competing interests to declare.

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Ethical approval

Not applicable.

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Author contributions

Oumarou Hama Hamadou: conceptualization, project administration, supervision, data curation, formal analysis, investigation, methodology, software, validation, visualization, original draft, writing - review & editing. Thomas Chenal: resources, writing - original draft, writing - review & editing. Olivier Pible: data curation, methodology, software, visualization, formal analysis. Guy-laine Miotello: investigation, methodology, data curation. Jean Armengaud: conceptualization, investigation, methodology, data curation, formal analysis, validation, visualization, writing - original

draft, writing - review & editing. Michel Drancourt: conceptualization, project administration, supervision, funding acquisition, validation, visualization, original draft, writing - review & editing.

Data availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium through the PRIDE partner repository with the dataset identifier PXD031786/Project DOI [10.6019/PXD031786](https://doi.org/10.6019/PXD031786) and PXD031802/Project DOI [10.6019/PXD031802](https://doi.org/10.6019/PXD031802) for extended data.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:[10.1016/j.ijid.2023.03.019](https://doi.org/10.1016/j.ijid.2023.03.019).

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