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BRIEF COMMUNICATION

Paleoserological detection of Coronavirus antigens in dental calculus of human remains dating from the beginning of the 19th century, French Ardennes

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Abstract

Objective: Vanishing viral RNA restricts our ability to detect ancient pathogens, so, we used paleo serological approaches to trace the dynamics of the Coronavirus in ancient populations.

Materials and Methods: We investigated 10 ancient dental calculus samples collected from a cemetery dated to the beginning of the 19th century and excavated in Charleville-Mézières. After paleoserum samples were extracted from dental calculus, paleoserology using mini-line-blot incorporating one alpha-Coronavirus (Coronavirus 229 E) and two beta-Coronavirus (Coronavirus OC 43, SARS-CoV-2) antigens and controls was completed by an automated Western blotting assay.

Results: Once appropriate controls had validated the data, mini-line-blot detected antibodies against the two beta-Coronavirus antigens in individuals US1300 and US1339, automated Western blotting confirming one beta-Coronavirus antigen for individual US1300 and an additional individual US1326.

Discussion: Combing mini-line blot and automated Western blot assays made it possible to detect immunoreactive immunoglobulin tracing circulation of Coronavirus in France at the very beginning of the 19th century.

KEYWORDS

automated Western-blot, beta-coronavirus, coronavirus, dental calculus, immunoglobulins, mini-line-blot, paleoserology

1 | INTRODUCTION

Human Coronaviruses (HCoV) form a group of RNA viral pathogens which recently rose to worldwide recognition during the COVID-19 pandemic caused by the deadly Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) (Pal et al., 2020). HCoVs have been documented in patients for almost 60 years after their initial isolation

from nasal discharges from patients with colds, along with step-by-step detection and isolation of additional Coronaviruses including HCoV-229E, HCoV-OC43, SARS-CoV, HCoV-NL63, HCoV-HKU1, and Middle East Respiratory Syndrome Coronavirus (MERS-CoV) causing colds and other clinical forms of infection with varying prognoses (Su et al., 2016). This impressive corpus of knowledge regarding HCoVs infecting populations for half a century, obscured the

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contrasting fact that the antiquity and dynamics of HCoV in the past populations has been poorly documented due to the fact that vanishing viral RNA significantly limits the possibility of directly detecting Coronaviruses in ancient specimens collected from individuals who have been buried.

To circumvent this limitation, we previously developed paleoserological techniques to specifically detect anti-Coronavirus antibodies in ancient dental pulp samples. This well-preserved specimen had been previously shown to encapsulate a blood drop and the immunoreactive antibodies it would contain (Oumarou Hama et al., 2020). Here, we developed a paleoserological technique to retrieve immunoreactive antibodies from ancient dental calculus and applied this new technique to the detection of anti-HCoV antibodies in several individuals who died at the beginning of the 19th century in France.

2 | MATERIALS AND METHODS

2.1 | Dental calculus specimens

The archeological site of Charleville-Mézières, located in the north-east of France (49°46' 19" N 4°42' 58" E) was discovered in 2021 during a preventive excavation at the northwest corner of the modern city of Charleville-Mézières. Historical and anthropological data indicated that at the beginning of the 19th century this site was a military hospital cemetery (Hubert, 1854), where more than 87 individuals had been buried. In the absence of any furniture (coffin or grave goods) associated with the human skeletons, three human bone samples were analyzed by radiocarbon dating (Poznan Radiocarbon Laboratory: F91 Us 1322 R_date: 170,30; F91 Us 1240 R_date: 195,30; F108 Us1291 R_date: 205,30) indicated that tombs are later than the second half of the 17th century, lacking more precision due to level of uncertainty linked to fluctuations of the calibration curve for this period. The radiocarbon signature was almost identical for all analyzed samples, suggesting a relative contemporaneity for the burials. Other graves exhibiting identical anthropological characteristics, were identified in 1994 in Charleville-Mézières at the Bazin High School. Archival study carried-out at the time proposed a parallel with the peaks in mortality recorded in the city between 1810 and 1817 (3540 deaths including a large number of soldiers of different nationalities) compatible with an excess of military mortality in 1813–1814 due to the retreat from Leipzig. The hundred individuals exhumed during the excavation were located in the immediate vicinity of the military hospital of Charleville-Mézières, inside the ramparts of the city, within an undefined funerary space, overflowing from the excavation area. Thirty-five graves were excavated, ten housing between two and 17 individuals. Later burials were deposited in several stages (they were first multiple and then collective) (Boulestin, 2019) indicative of several timely related mortality crises; an hypothesis confirmed by the observation of a lack of overall row organization but instead alignments, often of three pits, individual or collective. On several occasions, overlaps were observed. The biological study indicated an absence of female subjects and a strong representation of young adolescents and older adults with growth disorders.

Traces of pulmonary infectious disease appeared within our corpus. The frequent literature mention of epidemic causes of death for these populations (referred as cholera, typhus, and “military suette”) prompted a paleomicrobiological study. Accordingly, after skeletons were exhumed and stored at room temperature for several months, the present study incorporated 20 teeth, collected from 10 individuals (Supplement Table 1) and transported to the Paleomicrobiology Laboratory at the Institut Hospitalo-Universitaire Méditerranée Infection (IHU) in Marseille, France, for further paleomicrobiological investigation. There, deposits of ancient dental calculus were recovered from the surfaces of the teeth using sterile Gracey curettes (Dentsply Sirona, Charlotte, North Carolina, USA) and collected into sterile Eppendorf tubes.

As for the regulatory affairs, the French law (Law n° 2001–44 du 17 janvier 2001 relative à l'archéologie préventive) stipulates that no authorization is required for the scientific investigation of ancient burials. Also, according to this law 2001–44, ancient human remains are considered as archeological remains, in the same way as other remains. The anthropologist and archeologist, because of their position, are free to take samples and analyze them for their scientific research by any techniques they may need.

2.2 | Protein extraction

Each dental calculus sample was separately immersed in 200 µL of ethylene diamine tetra-acetic acid (EDTA), sonicated 5×1 min at 6000 J (5510 BRANSON, Danbury, USA) and incubated at 4°C for 48 hours under agitation. Sonicated samples were centrifuged for 30 min at 17,000g at room temperature and supernatants (referred to as EDTA fractions) were stored at room temperature. Pellets were washed twice with 200 µL of sterile distilled water, suspended in 100 µL of 50 mM ammonium bicarbonate solution (pH = 7.4) and incubated for 48 h at 75°C. The samples were centrifuged at 17,000g for 30 min at room temperature and the supernatants (called bicarbonate fractions) were collected and stored at –20°C. For each dental calculus specimen, the EDTA fraction and the bicarbonate fraction were pooled and dialyzed twice using Slide-Alyzer K2 MWCO dialysis cassettes (Pierce Biotechnology, Rockford, IL, USA) with 1 L of dialysis solution (50 mM ammonium bicarbonate) for 4 h, followed by the second one-liter dialysis overnight to extract the dental calculus proteins. Samples were then lyophilized for 24 h, suspended in 30 µL of phosphate buffered saline (PBS, ThermoFisher, Paisley, UK) and total proteins were dosed using a Qubit assay following the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA), as previously described (Oumarou Hama et al., 2020).

2.3 | Mini-line-blot assay

AntiHCoV immunoglobulins were tested using a mini-line blot assay incorporating inactivated alpha-Coronavirus 229 E and beta-Coronavirus OC43 and SARS-CoV-2 as antigens; skimmed milk as a negative control, and *Staphylococcus aureus* as a positive control

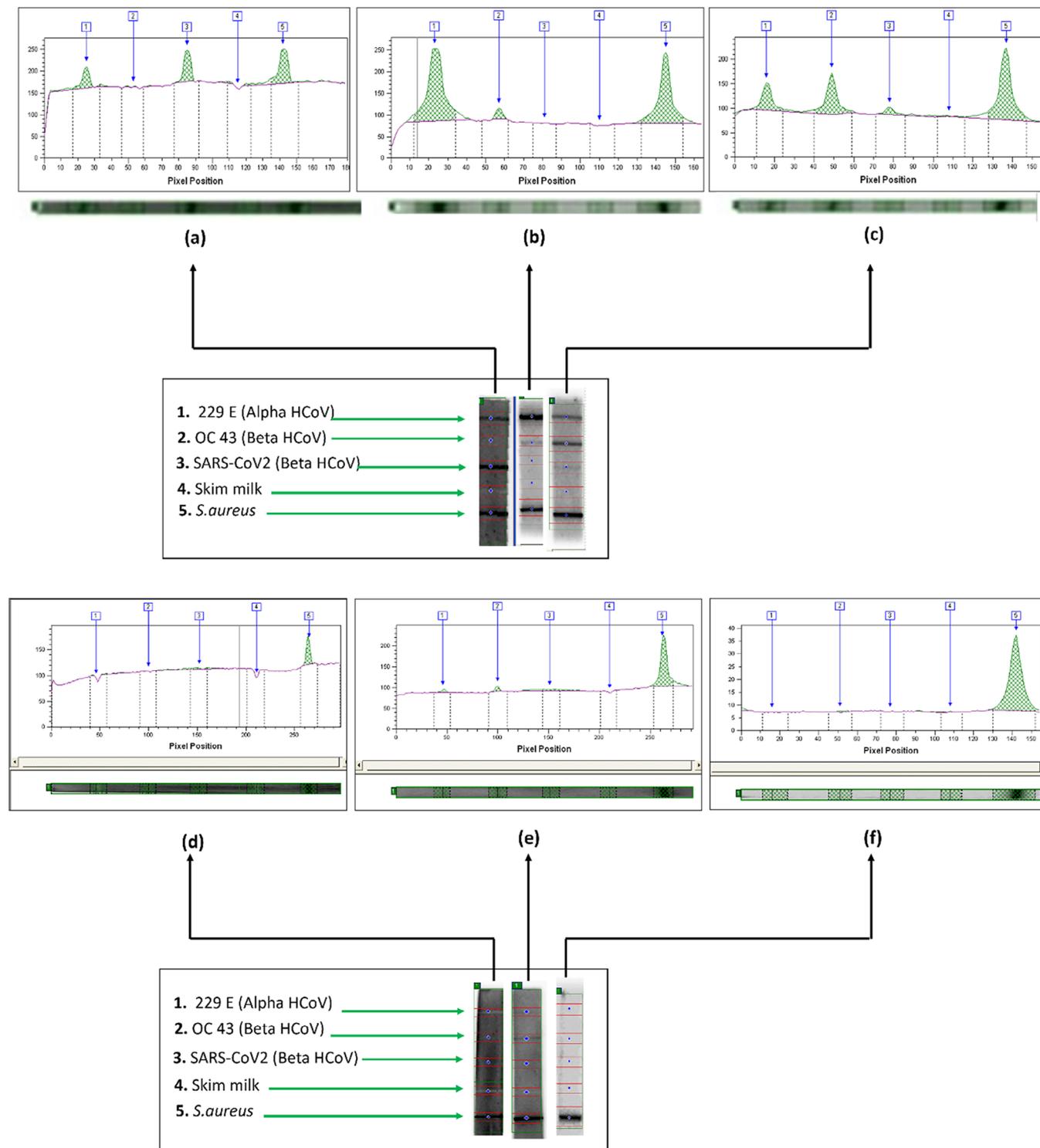


FIGURE 1 Mini-line-blot assay results for positive and negative control sera, (a) SARS-CoV-2-positive control serum, (b) 229 E-positive control serum, (c) OC43 positive control serum, and (d, e, f) negative control sera.

(Oumarou Hama et al., 2020). HCoV 229 E, OC43, and SARS-CoV-2 antigens were cultured and heat-inactivated in the NSB3 laboratory at IHU, as previously reported (Edouard et al., 2021). Antigens were deposited on a nitrocellulose membrane (Bio-Rad, Marnes-la-Coquette, France) using a pen, membranes were saturated in 5% PBS-milk for 1 h, incubated with 0.2–1 μL of modern leftover serum or 30 μL of paleoserum previously diluted at 1/1000 in a 0.5% saturation

solution and incubated for 2 h at room temperature on a rocker. Following three washes (3 X 5 min) with PBS-Tween, membranes were incubated with 1 mL of peroxidase-conjugated anti-human IgA/IgG/IgM antibody (Jackson Immuno Research, Ely, UK) diluted at 1/1000 in PBS-milk 0.5% for 2 h and washed three times (3 X 5 min) with PBS-Tween. Serological reactions were observed and quantified using the Fusion FX chemiluminescence imaging system connected to

ImageQuant TL software (Witec AG, Sursee Switzerland) and every mini-line-blot strip was individually read with reference to positive control and the skimmed milk negative control of the same strip (Oumarou Hama et al., 2020). Accordingly, the mini-line-blot assay was validated using three modern positive leftover sera collected from PCR-positive patients for each one of the three tested HCoV. Five leftover sera collected from IHU patients who tested PCR negative for each one of the three HCoVs were used as negative controls. These control serums were diluted at 1/1000 in PBS and Tween 80–0.5% skimmed milk.

2.4 | Automated western blot

In order to confirm the positivity of paleo serums, the JessTM Simple Western system and Jess 12-230-kDa separation module (SM-W004) were used according to the manufacturer's recommendations (ProteinSimple, San Jose, CA, USA) as previously described (Edouard et al., 2021). HCoV OC43 and SARS-CoV-2 antigens were mixed with 0.1X sample buffer and 5X fluorescent master mix to a final concentration of 0.25 µg/µL, fluorescent molecular weight markers and 400 mM dithiothreitol (ProteinSimple). This preparation was denatured at 95°C for 5 min. Ladder proteins (12-230-kDa PSST01EZ) and antigens were separated in capillaries upon migration through a separation matrix at 375 V. The ProteinSimple proprietary photo-activated capture chemistry was used to immobilize the separated viral proteins onto the capillaries.

Paleoserum samples diluted at 1/2 and 1/20 in the saturation buffer were added and incubated for 60 minutes. After a washing, HRP-conjugated human anti-IgG/IgM/IgA antibodies (Jackson Immuno Research, Ely, UK) diluted at 1/500 were added for 30 minutes. Chemiluminescence development was performed with peroxide/luminol-S (ProteinSimple). Digital chemiluminescence images of the capillary were captured with Compass Simple Western software (version 4.1.0, Protein Simple) which automatically calculated heights (chemiluminescence intensity), area and signal-to-noise ratio. The results were visualized as electropherograms representing the peak chemiluminescence intensity and as a lane view from the chemiluminescence signal detected in the capillary. An internal system control was included in each series (Edouard et al., 2021).

3 | RESULTS

3.1 | Mini-line-blot assay

The three modern positive control leftover serums reacted specifically against homologous antigens and against *S. aureus*, but not against skimmed milk and the five modern negative controls reacted only against *S. aureus* (Figure 1). Further, none of the 10 paleosera extracted from 10 ancient dental calculus samples, reacted against skimmed milk and nine paleosera reacted to *S. aureus* (Figure 2). The one which did not react exhibited a low concentration of protein

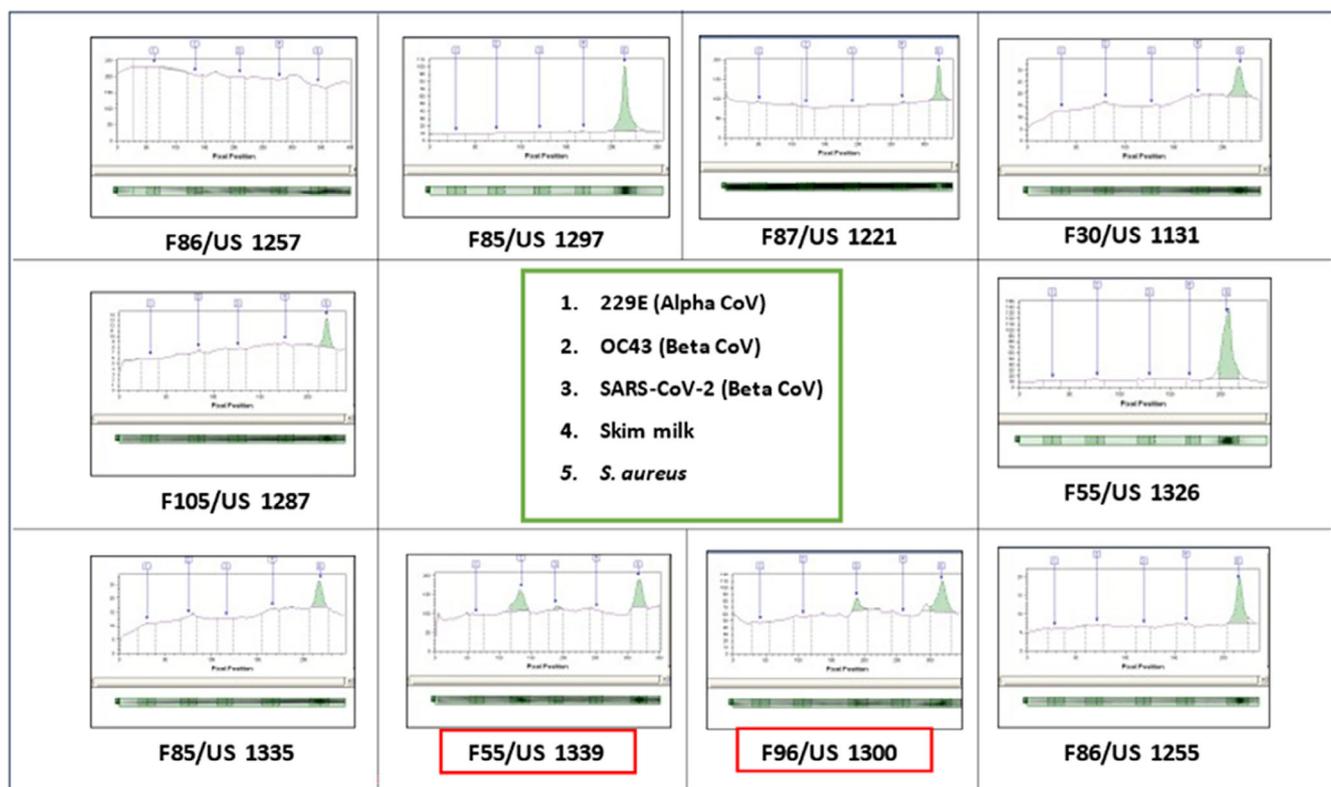


FIGURE 2 Mini-line-blot assay results for 10 paleosera extracted from 10 dental calculus samples collected from 10 individuals exhumed in the 19th century site of Charleville-Mézières, France. Two samples (red squares) were found positive by quantitative analysis by comparison with negative and positive controls.

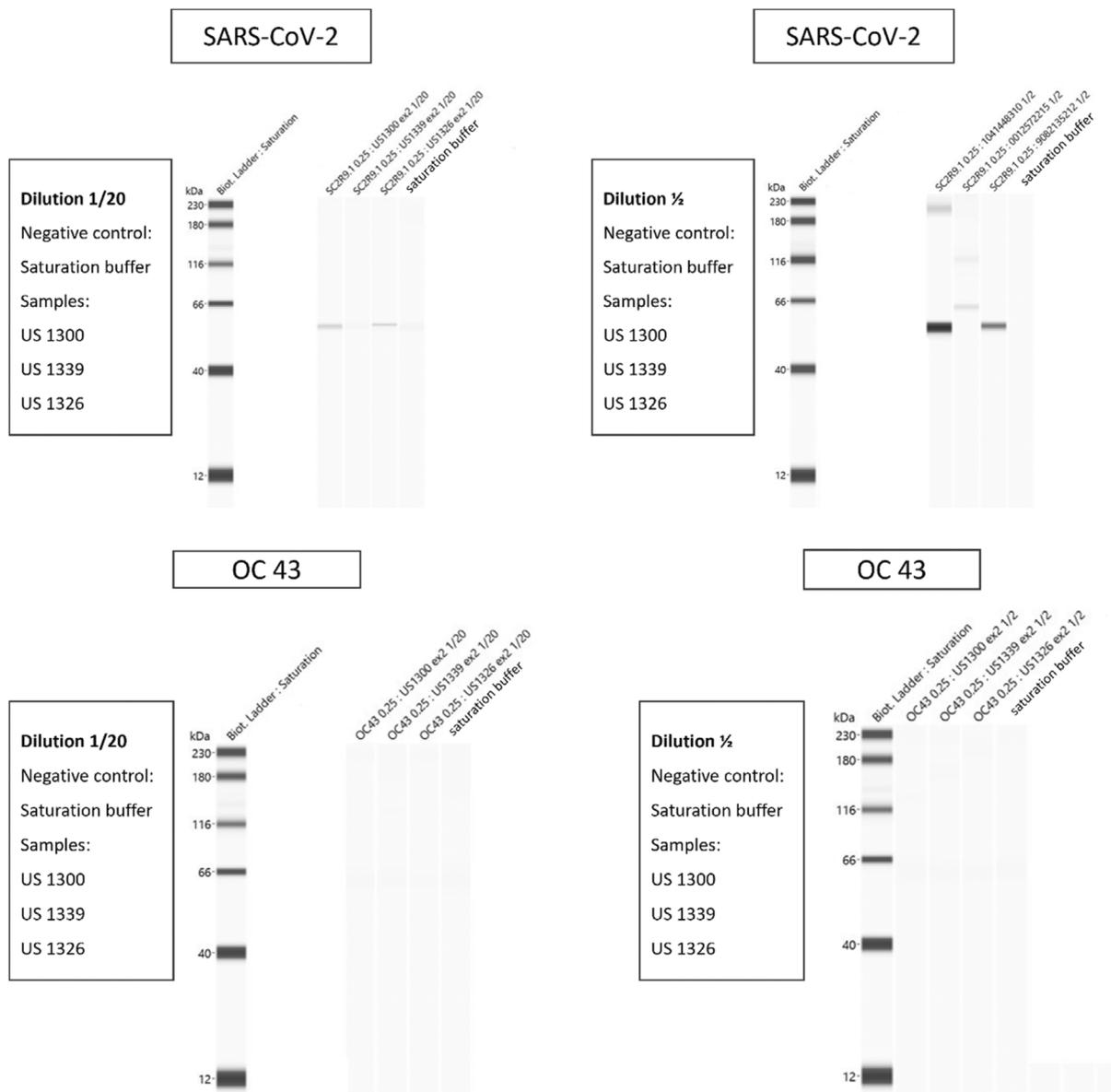


FIGURE 3 Automated Western blot detection of antibodies directed against anti-beta Coronavirus SARS-CoV-2 and OC43 in three paleosera extracted from three dental calculus samples collected from three individuals US1300, US1326 and US1339 exhumed in the 19th century site of Charleville-Mézières, France. Antibodies found positive by quantitative analysis by comparison with negative and positive controls obtained without any modification using Compass Simple Western software (version 4.1.0., Protein Simple) which is automatically calculating chemiluminescence intensity as well as signal / noise surface ratio. This figure exhibits visible bands against SARS-CoV-2 antigen but not visible band against OC43 antigen. One of the bands featured in both 1: 2 and 1: 20 diluted paleosera against SARS-CoV-2 antigen, is compatible in band size with the SARS-CoV-2 nucleocapsid and has therefore been interpreted as such. Extra-band of seemingly 230 kDa which is visible in US1300 sample (1: 2 dilution) was not interpreted, as it could be a nonspecific reaction against one of the molecular weight fluorescent standards.

<0.01 $\mu\text{g}/\mu\text{L}$ (Supplementary Table 1). Among these nine paleosera specimens, one paleosera sample, US 1300, reacted against SARS-CoV-2 antigen and one paleosera, US 1339, against the HCoV OC43 antigen (Figure 2).

3.2 | Automated western blot assay

In this assay three paleosera specimens, US1300, US 1339 and US1326, were tested against HCoV major protein nucleocapsids

(around 57 kDa) and Spike (around 217 kDa), as previously described (Edouard et al., 2021). When the negative controls, comprising a saturation buffer, and one negative modern leftover serum sample remained negative, no reactivity was observed against Coronavirus OC43 antigens (Figure 3). However, paleosera US1300, diluted 1/2 and 1/20, reacted against SARS-CoV-2 nucleocapsid antigen. Paleosera US1326, diluted 1/2 and 1/20, reacted against the SARS-CoV-2 nucleocapsid antigen, whereas paleosera US 1339, diluted 1/2 and 1/20, yielded no reactivity (Figure 3).

4 | DISCUSSION

We are reporting on the detection of anti-HCoV antibodies in individuals buried at the very beginning of the 19th century, in France. The negativity of the negative controls validated the data reported here. Although the serological methods used in this work, namely mini line blot and automated Western blot, have been previously reported to explore paleoserum samples recovered from ancient dental pulp specimens (Oumarou Hama et al., 2020; Oumarou Hama et al., 2023), the paleo serums here described have been uniquely recovered from ancient dental calculus specimens. Indeed, present study was built on previously reported data indicating the presence and characterization of immunoglobulins, including immunoglobulins reactive against some oral *Streptococcus* and *Actinomyces* pathogens in modern-day dental plaque (Cole et al., 1981; Tatevossian & Newbrun, 1981; Taubman et al., 1978). Furthermore, ancient dental calculus has been previously reported as a source of ancient proteins, including ancient host proteins and immunoglobulins (Warinner et al., 2014). This present study is nevertheless the seminal one demonstrating the immunoreactivity of such ancient, dental calculus-sourced immunoglobulins.

In this study, ancient immunoglobulins were observed to react with beta-Coronavirus OC43 and SARS-CoV-2 and to be more closely related to latter pathogen, following confirmatory results obtained by automated Western-blot assay which detected immunoreactive immunoglobulins against the SARS-CoV-2 nucleocapsid. However, the nucleocapsid has a high immunogenic power, being abundantly expressed during viral infections with cross-reactivities among beta HCoVs (Maache et al., 2006). In line with our own experience, the previous study demonstrated that cross-reactivity may depend on the type of serum studied (Che et al., 2005). Therefore, the present data did not allow us to further specify the HCoV to which these individuals had been exposed, a task which would require direct detection of the virus, most probably by appropriate paleoproteomic approaches, given that viral RNA may have vanished from more than 200-year-old, buried specimens. In particular, the present data do not specify SARS-CoV-2 as the specific HCoV to which these individuals were exposed. Also, the prevalence of exposure cannot be extrapolated from data reported here, due to the limited number of tested individuals; and unknown sensitivity in the particular context of paleoserology; the paleoserological methods being pioneeringly applied to ancient dental calculus.

Nonetheless, these results added to previous demonstrations of the circulation of an apparent beta-HCoV in soldiers who died in 1914 (Ramassy et al., 2022), predating these preliminary data by more than one century. Also, HCoV is one more pathogen to be added to the list of pathogens affecting populations at the beginning of the 19th century, after typhus caused by *Rickettsia prowazekii* and *Bartonella quintana* septicemia have been detected in soldiers returning from Russia during the episode referred to as the "Retraite de Russie" and buried in Vilnius, Lithuania (Raoult et al., 2006).

In conclusion, data here reported regarding the circulation of one beta-HCoV on individuals, presumably largely soldiers, at the beginning of the 19th century in France, indicate that ancient dental calculus

should be added to the list of ancient specimens suitable for the recovery and testing of ancient, immunoreactive immunoglobulins.

AUTHOR CONTRIBUTIONS

N. Merrouche: Conceptualization (lead); funding acquisition (lead); investigation (lead); visualization (lead); writing – original draft (lead). **S. Edouard:** Conceptualization (lead); funding acquisition (lead); visualization (lead); writing – review and editing (lead). **H. Oumarou Hama:** Conceptualization (lead); funding acquisition (lead); visualization (lead); writing – review and editing (lead). **D. Gucker:** Investigation (lead); resources (lead); writing – review and editing (lead). **S. Thiol:** Investigation (lead); resources (lead); writing – review and editing (lead). **N. Orain:** Investigation (lead); validation (lead). **G. Aboudharam:** Conceptualization (lead); funding acquisition (lead); validation (lead); writing – review and editing (lead). **M. Drancourt:** Conceptualization (lead); funding acquisition (lead); visualization (lead); writing – review and editing (lead). **E. Terrer:** Conceptualization (lead); funding acquisition (lead); validation (lead); writing – review and editing (lead).

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CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

All data are available.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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