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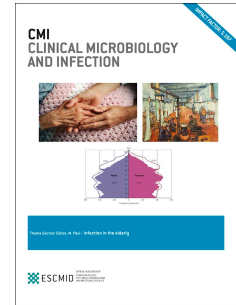
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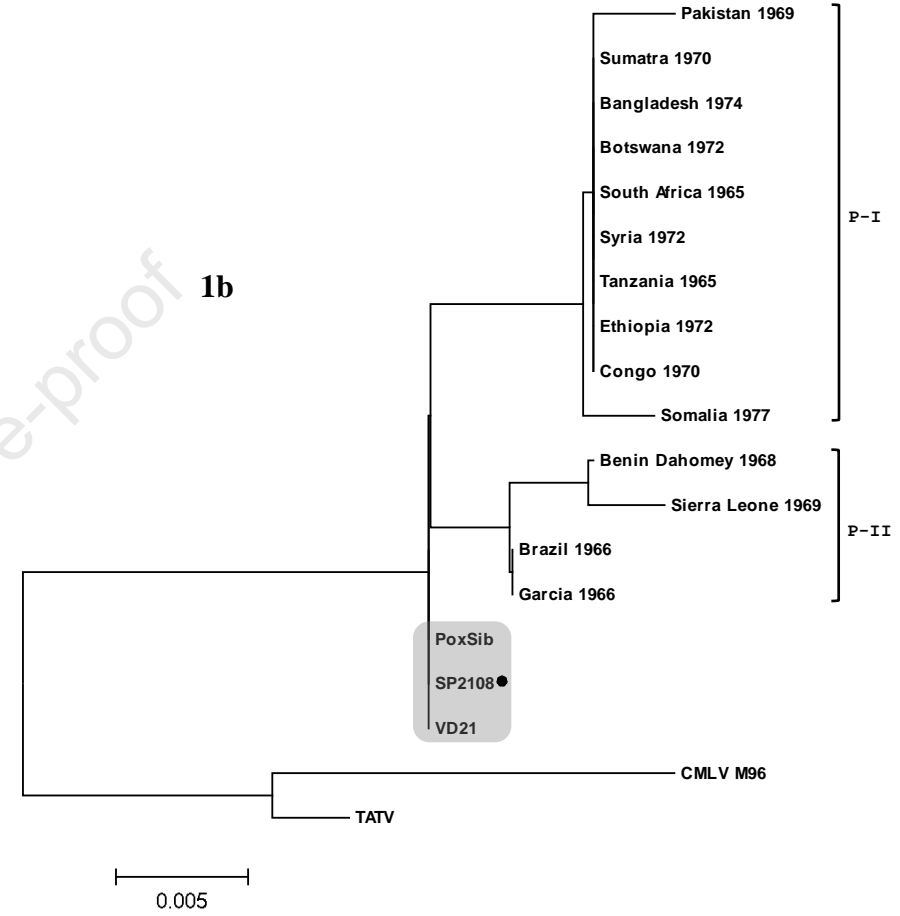
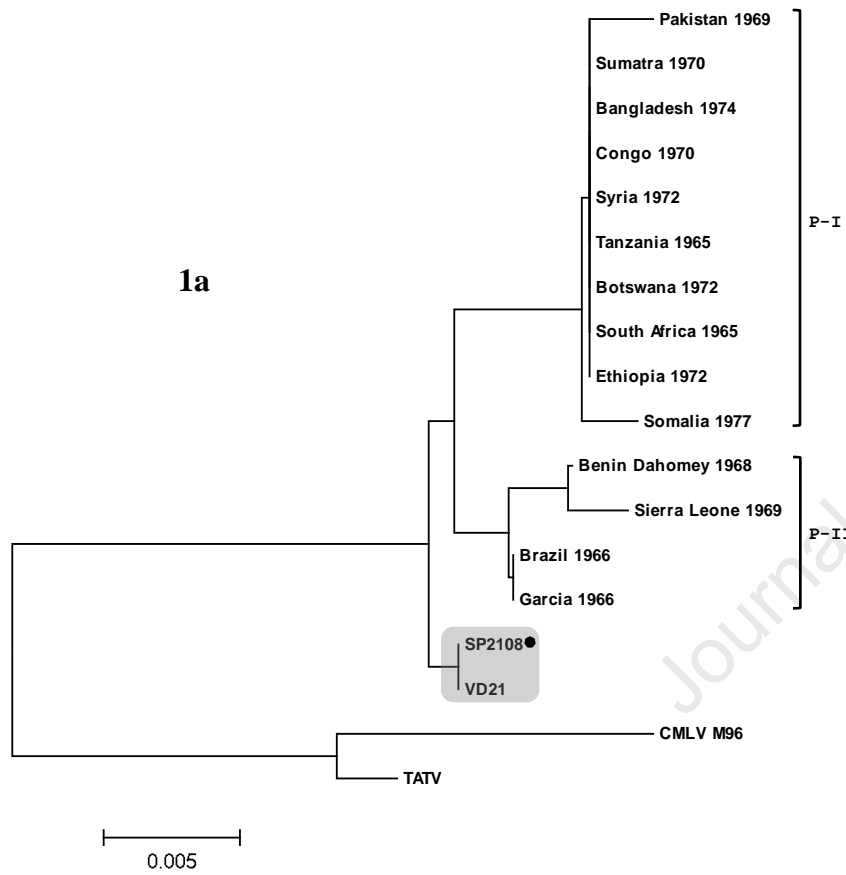
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1 Letter to the Editor

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3 **Variola virus DNA in skeletal remains, 17th-18th centuries,**
4 **southeastern France**

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25 *To the Editor,*

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27 Smallpox is one of the most devastating diseases known to mankind. Its deadly hold,
28 now vanished from the face of the earth thanks to a large-scale eradication campaign launched
29 by the World Health Organization (WHO) in 1967, has since become a subject of medical,
30 epidemiological and historical interest for the scientific community [1]. Ancient biological
31 remains have been investigated for variola virus (VARV) DNA, broadening our knowledge
32 on its evolutionary history in medieval / modern Northern Europe and modern Siberia [2-4];
33 however, we are still unable to estimate efficiently its presence in ancient urban or rural
34 populations from Western Europe.

35 Our study, stemming from previous palaeomicrobiological investigations performed
36 on contemporary foetus and infants possibly exposed to treponemal disease [5], aimed to
37 analyse 26 subjects excavated from the chapel of “Saint-Laurent de la Capelette” in Marseille
38 city, southeastern France (see Supplementary material, **Historical and archaeological**
39 **context** and **Table S1**). We carried out macroscopic and molecular analysis to evaluate the
40 prevalence of smallpox infection among this osteoarchaeological corpus dating back to the
41 mid-17th- early-18th centuries.

42 Bone samples (small cranial and costal elements) were collected and fully documented before
43 proceeding to molecular investigations. VARV DNA presence was investigated using several
44 PCR detection systems (<150 bp) targeting A38R, B7R, A30L, and E9L genomic regions; a 430
45 bp PCR amplification (E9L) to analyze the fragmented nature of the viral genome was also
46 performed (see Supplementary material, **Methods** and **Table S2**). Extensive precautions were
47 taken at each step of the study to meet with the ‘authenticity criteria’ expected for ancient DNA
48 studies [5]. No studies on modern smallpox were ever conducted in our facilities, and positive

49 controls were never used; negative controls (sterile water) were included systematically in
50 extraction and DNA amplification procedures.

51 Remains of one individual only (SP2108, child 1-4 year-old at the time of death, fairly
52 complete skeleton except for the skull; costal fragment tested) yielded positive PCR signals;
53 the four detection systems used gave amplicons of expected size on agarose gel
54 electrophoresis, whereas no signal was observed for the longer amplification assay
55 highlighting an extensive degradation of the viral genome.

56 Sequences analysis confirmed the VARV origin of amplified material, and revealed strict
57 identities with partial sequences obtained from Siberian [2] and Lithuanian [3] mummies
58 dating from similar periods (17th-18th centuries; PoxSib and VD21 isolates, respectively) (see
59 Supplementary material, **Figure S1**). Phylogenetic and temporal analysis of concatenated
60 sequences confirmed the divergent position of these 300-year-old VARV sequences, distinct
61 from 20th century P-I and P-II clades [2,3] (**Figure 1**, and Supplementary material, **Figure**
62 **S2**).

63 Our findings are consistent with smallpox being a Eurasian pandemic at this time,
64 spreading through the Ancient Continent via trade, migratory, exploratory and invasion routes
65 [1-4]. It echoes previous results, to the extent that the French SP2108 strain could either be an
66 ancestor of the 20th century VARV strains, or a member of an ancient lineage with no
67 connections, however, to the recent times outbreaks strains [2].

68 Importantly, above results entail also the possibility of bone samples from
69 archaeological context as a substrate for VARV molecular detection, the bone marrow being a
70 known site of replication for the virus, thus freeing palaeomicrobiologists from the necessity
71 of using mummified and/or frozen ancient remains to investigate this pathogen. Of note, the
72 skeleton of this young child displayed no signs of pathological conditions such as periosteal
73 remodelling or *osteomyelitis variolosa* (see Supplementary material, **Figure S3**).

74 In the present case, there is no reason to assume that this specimen was contaminated
75 by any other than the natural ways, as opposed to voluntary transmission by inoculation, since
76 the advent of this practice in modest populations did not occur in southeastern France until the
77 second half of the 18th century [1,6]. On the same note, the first immunization session in
78 Marseille took place only on March 27, 1801 at the “Hospice de la Charité”, involving
79 cowpox virus [6]. In the deteriorated sanitary context of the 17th and 18th centuries, smallpox
80 was a classic companion of Western populations, whether they were poor or favoured. As an
81 endemo-epidemic disease, it affected mainly children under 5 years of age and caused a high
82 mortality among them [6]. The parish archives of the “Saint-Laurent dite la Capelette” chapel
83 show the recurrence of young children burials, especially in the early 18th (see Supplementary
84 material, **Methods** and **Table S3**), with the occasional occurrence of a greater number of
85 losses among adults, that could result from more violent epidemic outbreaks. As pointed out
86 by P. Darmon, contrary to plague, “smallpox, in a much more diffuse way, decimated
87 consistently and piercingly by small touches, eliminating the generations at the base without
88 really slashing into the mass” [6]. Considered as a common and inevitable scourge, public
89 health policies of the time struggled little against its omnipresence and did not classify it as
90 part of quarantine diseases. The presence of smallpox throughout the year, with a mortality
91 pattern that staggered over the four seasons, did not lead to the use of mass burials, which
92 explains why this infected specimen was found in a normal burial context (parish church) [1,
93 6].

94 The uncovering of conserved, though fragmented, VARV DNA in 300-year-old bone
95 samples of a child's skeleton identified in south France adds clues to the dispersion of
96 smallpox in past populations; subsequent investigations are now expected.

97

98 **Transparency declaration**

99

100 **Author contributions**

101 All authors have made substantial contributions to the work. Study concept and design: AM, YA,
102 PB. Data acquisition: AM, YA, BS, SP, BP, MS. Analysis and/or interpretation of data: AM,
103 YA, PB. Drafting the manuscript: AM, PB.

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105 **Conflict of interest**

106 The authors declare that there are no conflicts of interest.

107

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111

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135 **Figure legends**

136

137 **Figure 1.** Neighbor-joining (NJ) phylogenetic trees generated using 20th and 17th-18th
138 centuries VARV, and animal orthopoxvirus partial sequences.

139 **a)** A38R, B7R, A30L, and E9L concatenated sequences (429 nt, SP2108 reference) (PoxSib
140 not included, no A38R sequence available).

141 **b)** B7R, A30L, and E9L concatenated sequences (325 nt, SP2108 reference).

142 20th century VARV P-I / P-II clades are shown. 17th-18th centuries VARV isolates (PoxSib-
143 Siberia/Yakoutia; VD21-Lithuania; SP2108-southeastern France, this study) are shaded. Scale
144 bar indicates nucleic acid substitutions per position.

145