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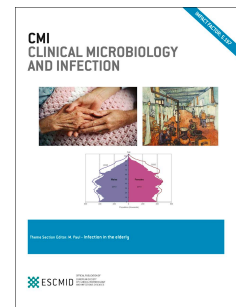
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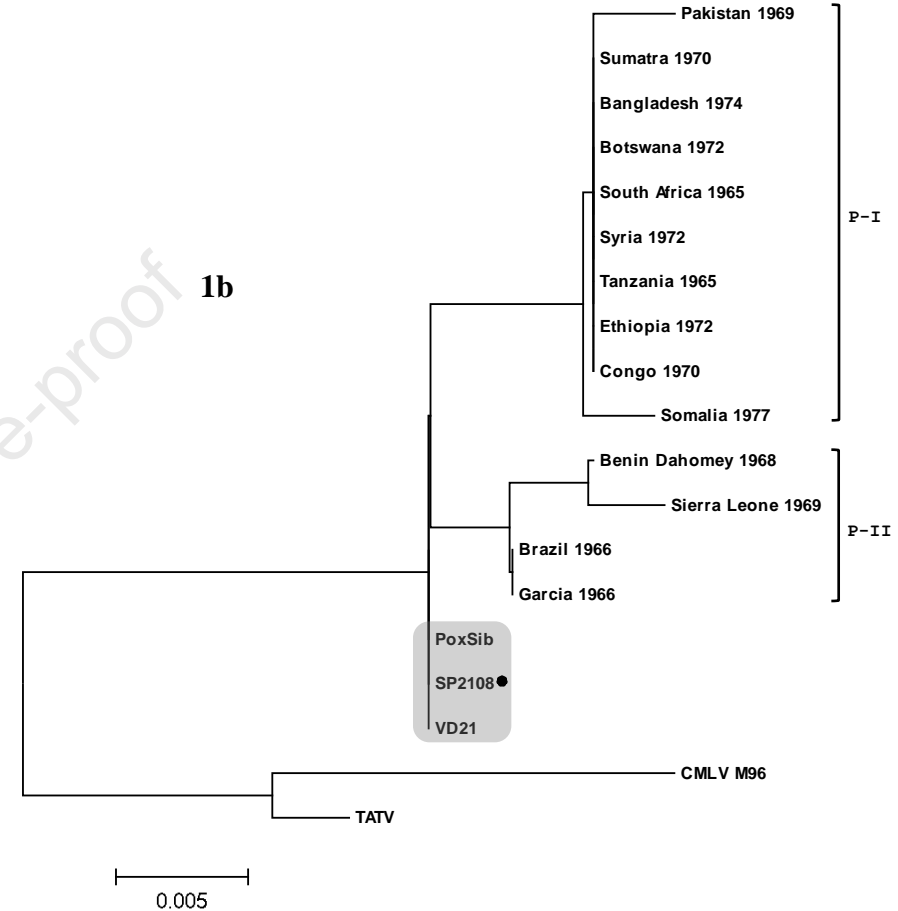
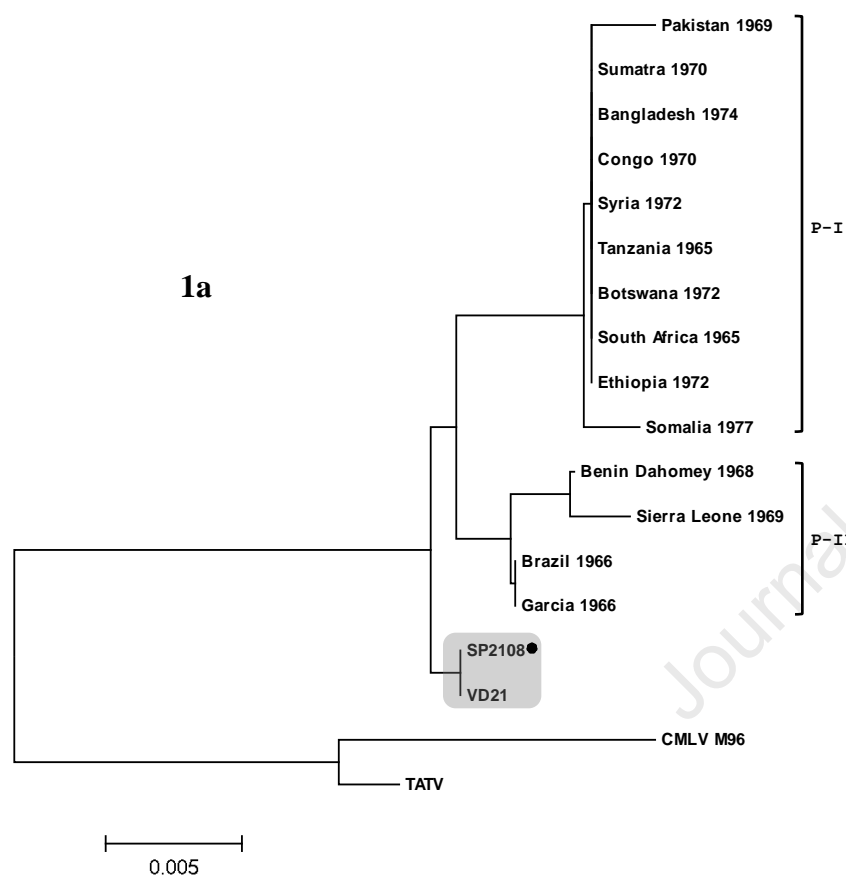
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Letter to the Editor

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

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To the Editor,

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

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

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

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

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

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

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

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

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

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

Transparency declaration

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

Conflict of interest

The authors declare that there are no conflicts of interest.

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Figure legends

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

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

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

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