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LETTERS TO THE EDITOR

No proof that typhoid caused the Plague of Athens (a reply to Papagrigorakis et al.)

The etiological agent of the Plague of Athens (430—426 BC) has been a hotly debated topic.[1] Recently Papagrigorakis et al.[2] claimed to have isolated typhoid fever DNA (caused by *Salmonella enterica* serovar Typhi) from three 2500-year-old teeth from putative plague victims. DNA amplifications from these teeth, which come from the ancient Kerameikos mass burial site in Athens, resulted in two fragments of DNA, both showing some similarity to previously published *Salmonella* species.

The authors' diagnosis is based on a similarity score, resulting from BLAST comparisons between their amplified fragments and published sequences. Specifically, the authors report a 7% divergence between their sequences and *S. enterica* serovar Typhi, and 8% divergence between their sequence and the next most closely-related *Salmonella* strain, *S. typhimurium*. Based on the closer match to *S. enterica*, the authors conclude that "[if] another, yet unknown pathogen... was the actual cause of the Plague of Athens, it would have to be closely related to *S. enterica* and definitely closer than *S. typhimurium*."[2]

This statement, however, is simply not true. Although the Athens sequence is indeed slightly more similar to *S. enterica*, the two cited *Salmonella* species are actually much more closely related to each other, with less than 1% divergence for the sequenced gene. In fact, if a simple phylogenetic analysis is performed, the ancient sequence is shown to fall outside both *S. enterica* and *S. typhimurium*, as well as several other *Salmonella* species (Figure 1). While this analysis confirms that the Athens sequence is possibly *Salmonella*, it demonstrates clearly that it is not typhoid (97% bootstrap value; Figure 1). Based on the evolutionary timescale inferred for *Salmonella* and *E. coli*,[3] the Athens sequence and typhoid would have shared a common ancestor in the order of millions of years ago.

While we cannot exclude the possibility that the Athens sequence is a previously unidentified infectious agent which caused the Plague of Athens, it is quite reasonable to assume that the sequence is actually that of a modern, free-living soil bacterium, a possibility that could have been explored by extracting DNA from surrounding soil samples as additional negative controls. What is certain is that the sequences obtained do not implicate typhoid as the cause of the plague.

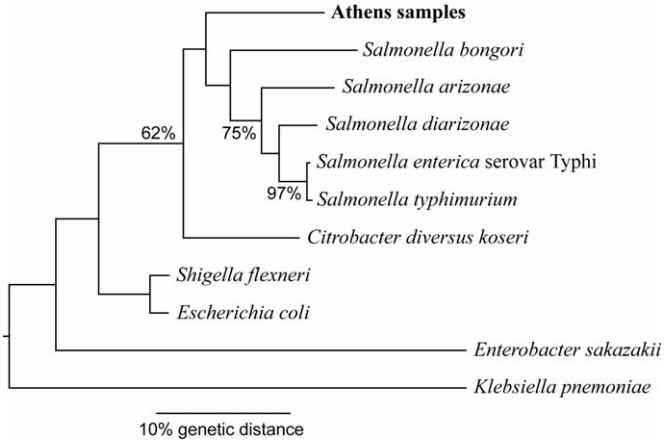

Figure 1 Neighbor-joining (NJ) tree constructed by PAUP* 4.0b10[6] using the HKY-Γ model of substitution (ts/tv = 2.4, Γ shape = 0.29; estimated using maximum likelihood (ML)) for 360-bp of the nitrate reductase gene. Bootstrap values are given for pertinent branches (10 000 replicate NJ trees using ML distances as above). Bacterial sequences other than the Athens sequence were obtained from GenBank, Washington University, St. Louis, USA (http://genomeold.wustl.edu/projects/bacterial/) and the Sanger Institute (http://www.sanger.ac.uk/Projects/Salmonella/).

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This study highlights one of the most significant and recurring problems in ancient DNA research: that of authentication of results. While many labs are now careful to use strict experimental controls (as outlined in Cooper and Poinar[4]), the recommendation of phylogenetic verification of results continues to be ignored, despite the relative simplicity and lack of expense of such tests, particularly in comparison to experimental procedures. It should be noted that many of the most embarrassing mistakes in the ancient DNA literature (including the incorrect report of dinosaur DNA in the early 1990s[5]) could have been avoided with a simple phylogenetic test.

Conflict of interest: No conflict of interest to declare.

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Insufficient phylogenetic analysis may not exclude candidacy of typhoid fever as a probable cause of the Plague of Athens (reply to Shapiro et al.)

The cause of the Plague of Athens (430—426 BC) has been debated among scientists, who have relied exclusively on Thucydides' historical narrations[1] to introduce several possible diagnoses.[2,3] The application of DNA analysis on skeletal remains taken from the Kerameikos mass grave[4] has been acknowledged as an ideal material that might provide clues for a definite evidence-based diagnosis of the epidemic.[5] Following a research methodology of proven accuracy and validity ('suicide' PCR),[6] it was shown by analysis of three genes (*osmC*, *clyA*, *narG*) that an ancient strain of *Salmonella enterica* serovar Typhi was present in the investigated dental pulp material of three putative victims of the plague, thus incriminating typhoid fever as a probable cause.[7]

Despite this evidence-documented approach, Shapiro et al.[8] have argued against the validity of these results. Through the application of a simple phylogenetic analysis of the published sequence of one gene (*narG*), Shapiro et al. concluded that, although the sequenced ancient DNA quite possibly corresponds to a *Salmonella* strain, this might not be typhoid. Instead, they argue that this sequence is more closely matched to other *Salmonella* species such as *S. bongori*, *S. arizonae*, and *S. diarizonae* rather than *S. enterica* Typhi. The authors base this assumption on an inferred evolutionary timescale of *Salmonella* and other related bacterial species. Shapiro et al. eventually make the assumption that the identified sequence most probably represents a modern and currently unknown free-living soil bacterium instead of an ancient one.

Against these arguments we must once again state the extreme preventative measures that were taken in our study[7] to exclude any possibility of environmental contamination. These included the absence of the pathogens themselves or a previously attempted extraction or PCR amplification of the target DNA sequences in the implemented laboratories, and also the 'suicide' PCR methodology that was followed, which excluded positive controls from this study. In addition, since possible environmental contamination is a major problem with ancient DNA studies as Shapiro et al. suggest, soil wash was actually used as a negative control in addition to DNA extracts from modern teeth. As is clearly stated in the original publication of our results, no product was yielded following the application of the same primers under the same laboratory conditions on the negative controls as well as on the soil sample washed off the ancient teeth,[7] thus excluding the possibility of any contamination of the investigated ancient material. Besides, *Salmonella* species do not survive for long in soil, which is typically regarded as a transitional environment for this pathogen prior to its infecting a host.[9] Even if the soil of the mass grave was indeed contaminated by a modern *Salmonella* strain, such as a close relative of *S. bongori*, *S. arizonae*, and *S. diarizonae* (which are naturally found in reptiles), as suggested by Shapiro et al.,[8] it would not be possible for any of these pathogens to survive during the long storage of the skeletal material and not be identified in the subsequently conducted investigation of the soil wash.

On the other hand, the application of phylogenetic models, as suggested by Shapiro et al.,[8] undoubtedly constitutes a powerful tool for the introduction of theoretical assump-